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EVALUATION OF DIETHYLENEGLYCOLDINITRATE (DEGDN) AND TWO DEGDN-CONTAINING COMPOUNDS

FINAL REPORT

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This study evaluates diethyleneglycoldinitrate (DEGDN) and two DEGDN-containing compounds, DIGL-RP and JA-2, for mutagenicity and carcinogenicity in mammalian cells in vitro. The potential mutagenicity of the three test articles was assessed by point mutation at the thymidine kinase locus in the mouse lymphoma cell (L5178Y TK+/-) forward mutation assay. The potential carcinogenicity of the three test articles was assessed by in vitro cell transformation as measured by anchorage-independent growth of Rauscher leukemia virus-infected rat embryos cell (RLV-RE). The results indicated that the three test articles contained direct-acting mutagen(s) which was shown by increased mutagenic activity without the addition of rat liver S-9 and the lack of enhanced mutagenic activity with the addition of the rat liver S-9 activation. Linear dose-mutation responses of statistical significance were detected with DEGDN, DIGL-RP and JA-2; DEGDN had the effect of a weak mutagen while DIGL-RP and JA-2 had the effects of a strong mutagen. The strong direct-acting mutagenic (continued)

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18. mouse lymphoma cell L5178Y, Rauscher leukemia virus-infected rat embryo cell (RLV-RE), point mutation, thymidine kinase locus, cell transformation anchorage-independent growth.

19. activity that was detected in DIGL-RP and JA-2 was induced by a component(s) other than DEGDN. The qualitative assessment of the three test articles for carcinogenicity by the RLV-RE cell transformation assay in vitro suggested that DIGL-RP and JA-2 may be potential carcinogens while DEGDN is probably not a carcinogen.

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EXECUTIVE SUMMARY

The nitrate ester, diethyleneglycoldinitrate (DEGDN) was introduced to replace nitroglycerin as a plasticizer for nitrocellulose in explosives used in military weaponry. There is limited information on the potential latent health hazards to personnel during the production, processing, packing and firing of DEGDN-containing munitions. In the absence of adequate data on the potential health hazards of DEGDN-containing munitions, the Department of Defense requested an evaluation of DEGDN and two DEGDN-containing solid propellants, DIGL-RP and JA-2, for mutagenic and carcinogenic activity in mammalian cells *in vitro*.

The three test articles, DEGDN, DIGL-RP and JA-2, were evaluated for mutagenicity by the point mutation assay at the thymidine kinase locus in mouse lymphoma cells (L5178Y) and for carcinogenicity by the cell tranformation assay in Rauscher leukemia virus-infected rat embryo cells (RLV-RE). Each test article was tested for mutagenicity at three or more concentrations with or without rat liver S-9 activation and for carcinogenicity at two concentrations with and without a promoter, 12-O-tetradecanoyl phorbol 13-acetate (TPA). Regression analyses and analyses of variance were conducted to establish the statistical significance of the data.

These studies show that DEGDN acted on the mouse lymphoma cells as a weak mutagen while both DIGL-RP and JA-2 affected the mouse lymphoma cells as strong mutagens. Each test article was observed to induce a statistically significant increase in the number of mutants with heritable and stable properties over and above the spontaneous mutation rate in the controls. DEGDN failed to induce an increase in the number of RLV-RE transformed cells while DIGL-RP consistently induced increased numbers of RLV-RE transformed cells with and without TPA. On the other hand, JA-2 was observed to induce RLV-RE tranformation only in the presence of the promoter, TPA.

Based on these studies, it was concluded that both DIGL-RP and JA-2 may be potential, serious health hazards while DEGDN may not pose the same level of health hazards for personnel exposed to or handling these test articles. Since DIGL-RP and JA-2 are complex mixtures, the identity of the specific active component(s) is not known, nor is it known whether the observed mutagenic activity is an additive or synergistic effect of multiple weak mutagens.

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INTRODUCTION

OBJECTIVE

- To evaluate the mutagenicity and carcinogenicity in mammalian cells in vitro of DEGDN and two DEGDN-containing solid propellants, DIGL-RP and JA-2.

SPECIFIC AIMS

- To determine the mutagenic activity of DEGDN, DIGL-RP, and JA-2, with and without rat liver S-9 activation on mouse lymphoma cells (L5178Y TK+/-).

- To determine the carcinogenic activity of DEGDN, DIGL-RP, and JA-2 with and without the addition of the chemical promoter 12-O-tetradecanoyl phorbol 13-acetate (TPA) by the Rauscher leukemia virus-infected rat embryo cell (RLV-RE) transformation assay.

BACKGROUND

Health hazards problem

The U.S. Department of Defense (DOD) is currently considering the broader utilization of a new propellant formulation which will use DEGDN in place of nitroglycerin. There is limited information on the potential health hazards of DEGDN and the DEGDN-containing solid propellants. This study was designed to evaluate the potential mutagenic and carcinogenic activity of the three test articles by exposing mouse lymphoma and RLV-RE cells in vitro.

Historical background

The historical background on the development and use of DEGDN, as well as other nitrate esters as co-energetic/plasticizer was reviewed by Holleman et al.[1] They found very little information on the carcinogenic, mutagenic and teratogenic potential of nitrate esters. They reported no information on in vitro studies on the mutagenicity and carcinogenicity of DEGDN and DEGDN-containing solid propellants.

In vitro mutagenesis

Several short-term mutagenicity assays utilizing pro- or eukaryotic cells have been developed and are being actively utilized for assessing the genotoxic effects of various synthetic and natural chemicals.[2,3,4,5] The genomic organization of an eukaryotic cell is more complex than that of a prokaryotic cell. Because of genomic complexity of mammalian cells, mutagenesis in cultured mammalian cells is more relevant to man than are bacterial, fungal, or insect systems. In addition, compared to whole-animal studies, cultured cells offer the advantage of ease of handling, low cost and rapidity of assaying for mutagens.[6]

The mouse lymphoma L5178Y cells for detection of point mutation at a specific gene locus, as represented by the heritable loss of thymidine kinase activity which is a metabolic pathway for DNA synthesis was selected as an assay for mutagenicity. The mouse lymphoma mutagenicity assay was first reported by Clive, et al.[4] and has been used by other investigators. By selecting a specific gene locus that can be readily detected, one can quantitate the effect of a mutagen on that locus. Thymidine kinase is an enzyme that is encoded by a specific gene which is involved in the synthesis of thymidine monophosphate (TMP). TMP occupies a unique position in DNA synthesis because it does not undergo significant conversion to other nucleotides. The cellular TMP pool is small and depletion of the pool can arrest DNA synthesis. Replacement of TMP in the DNA with a lethal TMP analogue can result in the death of the cell. 5-Bromodeoxyuridine (BUdR) monophosphate and trifluorothymidine (TFT) monophosphate are lethal analogues of TMP which result from phosphorylation of BUdR or TFT by the "salvage" enzyme TK. Mutant cells in which TK activity is absent (TK-/-) thus do not suffer any ill effects when they are exposed to BUdR or TFT. By selecting mutant cells heterozygous for the thymidine kinase gene locus (TK+/-) which are sensitive to BUdR or TFT, one can measure the forward mutation at that gene locus, i.e. TK+/- ⟶TK-/-, by counting the number of cloned colonies that form in media containing BUdR or TFT following exposure to a mutagen. Following standardized procedures with adequate controls, one can quantitate the mutagenic potential of various chemicals.

Although each mammalian cell system has advantages and disadvantages, the major advantages in using the mouse lymphoma cells include high sensitivity, rapid cell growth with high cloning efficiency, growth of the cells in suspension culture, and quantitation of the cytotoxic effect of a genotoxic agent.

In vitro carcinogenesis

An in vitro carcinogenesis assay is a short-term mammalian cell transformation assay for the detection of potential chemical carcinogens in vitro. Although long-term animal studies provide the most accepted and definitive proof in testing potential carcinogens, the costs in money and time often make it impractical to test a wide variety of compounds. When Berwald and Sachs[7] reported that cells in culture could be transformed by chemical carcinogens, it opened the way for the development of several mammalian cell systems for the identification of potential carcinogens. The appeal of the in vitro cell system was that the cells had properties characteristic of tumorigenic cells in vivo. Some of the characteristics common to transformed cells are loss of density-dependent inhibition of cell division, resulting in foci of cell growth that are disorganized or in criss-cross fashion, and anchorage-independent growth. Cell transformation assays, unlike mutagenic assays, can detect potential carcinogens that do not alter the genomes but may act through epigenetic mechanisms. Thus, this class of assay can detect a wider range of potential health hazards than the mutagenic assays and is a valuable intermediate assay between mutagenic assays and whole animal exposure studies.

Cell transformation assays can be divided into three major categories. The first category uses primary or early passage cells; the second uses established cell lines; and the third uses cells that have been infected with a virus. Each cell transformation category has utilized several types of cells and each has its advantages and disadvantages. Recently, several investigators have compiled, compared, and evaluated the results of the different assays.[8,9] These investigators found that each assay as a group showed good correlation with in vivo carcinogenic activity but the consensus was that there is no single cell-transformation assay that could be recommended as the assay of choice.

The RLV-RE transformation assay was first developed and used by Freeman et al.[10] These investigators observed that this assay was highly sensitive and could detect various classes of chemical carcinogens. Traul et al.[11] initiated a new RLV-RE cell culture which was found to be equally useful and included the additional end point of anchorage-independent growth, which was found to be a more consistent end point that correlated well with in vivo tumorigenicity.

The RLV-RE cell transformation assay was selected for this study because: 1) this assay has been used extensively to test a large body of chemicals and has shown a high concordance with the Environmental Protection Agency (EPA) list of positive carcinogens; 2) rat embryo cells have retained some metabolic capability to activate procarcinogens without addition of an exogenous metabolic agents or feeder layer; 3) the RLV-RE assay has low spontaneous cell transformation; 4) the RLV-RE assay is highly sensitive for detection of a spectrum of chemical carcinogens; and 5) the major end point for this assay is clonal growth in soft agar media, which is a more reliable and accurate proof of cell transformation than morphological changes alone.

Disadvantages of the RLV-RE cell transformation assay are: 1) the assay is labor intensive, 2) cell cultures must be continuously maintained for several months, and 3) the assay data can be used only for qualitative and not quantitative assessment of potential carcinogenic activity because transformed anchorage-independent cells can be lost during the several medium changes and cell passages.

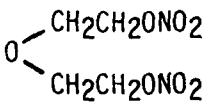
MATERIALS AND METHODS

TEST ARTICLES

The test articles that were received from Radford Ammunition Plant were diethyleneglycoldinitrate (DEGDN) and two DEGDN-containing solid propellants, DIGL-RP and JA-2. The chemical and physical properties of DEGDN were reviewed by Holleman, et al.[1] and the chemical structure is provided on Table 1. The military specifications for the solid propellants DIGL-RP and JA-2 are from DOD-P-63492 (AR) 30 June 1983, and DOD-P-63493 (AR) 14 June 1983, respectively. The chemical composition of the solid propellants are provided in Table 1. DEGDN was received as a liquid with 15% acetone added to desensitize the compound while DIGL-RP and JA-2 were received as solids. DIGL-RP and JA-2 were pulverized under liquid nitrogen at Letterman Army Institute for Research before they were solubilized in 100% dimethyl sulfoxide (DMSO).

TABLE 1. STRUCTURE OF DIETHYLENEGLYCOLDINITRATE (DEGDN) AND COMPOSITION OF TWO DEGDN-CONTAINING SOLID PROPELLANTS DIGL-RP AND JA-2.

(A) Structure of diethyleneglycoldinitrate (DEGDN)



(B) Composition of solid propellants DIGL-RP and JA-2

Ingredients	DIGL-RP* % w/w	JA-2** % w/w
1. Nitrocellulose	62.50	59.50
2. Diethyleneglycoldinitrate	36.70	24.80
3. Nitroglycerin	--	14.90
4. N-Methyl-N' N' Diphenylurea	0.45	0.70
5. Ethyl Centralite	0.25	--
6. Graphite	0.04 to 0.05	0.04 to 0.05
7. Magnesium Oxide	0.04 to 0.05	0.04 to 0.05
8. Graphite Glaze	0.20 Max.	0.20 Max.

Composition from military specification:

* DOD-P-63492

** DOD-P-63493

The purity of DEGDN was calculated to be 99.05% based on the nitrogen content of 14.15%. The chemical analysis of DEGDN was determined by Hercules Inc., Radford Army Ammunition Plant, Radford, VA. The composition of the DIGL-RP and JA-2 was from military specifications and no chemical analysis was conducted at LEHR.

All stock solutions were prepared in 100% DMSO and working solutions were prepared in tissue culture media supplemented with DMSO. The DEGDN/acetone stock was prepared as 10% v/v solution, while DIGL-RP and JA-2 stocks were prepared as 20 mg/ml solution. Stock solutions were stored in dark amber vials at 0°C. In preparation of working solutions, the stock solution was thoroughly mixed by pipetting and then a specific volume was pipetted into Fischer's media which was supplemented with DMSO to make the final DMSO concentration equal to 4% v/v. Each test sample had a 4% v/v final DMSO concentration. All laboratory procedures were conducted in a laminar flow hood.

STANDARD MUTAGENS

Promutagen

The 2-acetylaminofluorene (2AAF) (2-acetamidofluorene No. 7015) was purchased from SIGMA Chemical Co., St. Louis, MO.

Mutagen

Ethylmethane sulfonate (EMS) (methanesulfonic acid ethyl ester No. M0880) was purchased from SIGMA Chemical Co., St. Louis, MO.

CHEMICALS AND BIOLOGICALS

The materials used included: Glycine, hypoxanthine, isocitric acid, methotrexate, 12-O-tetradecanoyl phorbol 13-acetate (TPA), beta-nicotinamide adenine dinucleotide phosphate (B-NADP), thymidine (Sigma Chemical Co.), 3-methylcholanthrene (3-MCA) (Eastman Kodak), Aroclor 1242 and 1254 (Foxboro Co.), Pluronic F-68 (BASF Wyandotte Corp), Dimethylsulfoxide (DMSO) (Mallinkrodt), Dextrose, L-glutamine, sodium pyruvate, penicillin-streptomycin mix, Fischer's media, Eagle's Minimum Essential media with Earle's salt, Hank's balanced salt solution, trypsin, trypan blue solution (Gibco), Agar (Mann Res Lab), Gentamicin sulfate (M.A. Products), Giemsa stain (MCB Reagents), and equine and fetal bovine serum (K.C. Biologicals).

MOUSE LYMPHOMA CELL L5178Y TK+/-

The mouse lymphoma cells (MLC) were received from Dr. D. Clive (Burroughs Wellcome Co.). These cells were propagated and aliquots of cells were cryopreserved in 50% Fischer's Medium, 35% horse serum and 15% DMSO. Cells frozen at comparable passage levels were used throughout each study. Ongoing cultures were maintained in Fischer's medium supplemented with 10% horse serum, 0.11 mg/ml sodium pyruvate, 1.5 mg/ml glutamine, 1.0 mg/ml Pluronic F-68, 200 units/ml penicillin and 200 µg/ml streptomycin and 2.0 mg/ml sodium bicarbonate. This was designated as F10P medium where the numerical designation was for the serum concentration. Prior to each assay, the frozen cells were rejuvenated and grown in F10P containing 3 µg/ml thymidine, 5 µg/ml

hypoxanthine, 7.4 μg/ml glycine and 0.1 μg/ml methotrexate for 24 hours (to cleanse the culture of TK-/-cells) followed by 24-hour incubation in similar media without methotrexate. The cells were grown in F10P medium thereafter. The cells were used in assay 4 days after treatment with methotrexate.

In the descriptions of methods used the percent of horse serum added to the medium forms part of the designation of that medium. Thus, F10P, F5P, and F0P contain 10%, 5%, and no horse serum, respectively.

PREPARATION OF RAT LIVER S-9

The procedure for preparation of rat liver S-9 is as previously described by Clive and Spector.[12] The Aroclor mix consisted of 333.3 mg of Aroclor 1242 and 166.8 mg of Aroclor 1254 in 2 ml of corn oil. Three male Sprague Dawley rats were injected intraperitoneally with 0.5 ml of the Aroclor mix per 200 gm body weight. Five days later the rats were sacrificed and the livers were aseptically removed and rinsed in ice-cold sterile 0.25 M sucrose solution. The chilled livers were homogenized under aseptic conditions in three volume equivalent sterile sucrose solution. The liver homogenate was centrifuged at 9000 x g at 4°C for 20 minutes. The pooled supernatant was placed in sterile vials and stored at -70°C. Each new lot of S-9 was tested for sterility and for activation of a known quantity of promutagen (2-AAF) to obtain a standard unit of activity.

MOUSE LYMPHOMA CELL FORWARD MUTATION ASSAY

This assay was separated into several steps as follows: cytotoxicity test, exposure, expression, cloning in semi-solid media, counting colonies and data anaylsis.

Cytotoxicity Test

Test compounds were initially tested to find the range of nontoxic to toxic doses and to determine the concentrations to be used in the final assay. In this test, the cells that were exposed to the compounds were monitored for their growth rate for 3 days and the relative growth rates were compared with the control. Appropriate solvent and negative controls were included at this time. The final assay usually included three concentrations, one at LD_{50}, one with slight toxicity and one non-toxic concentration.

Exposure

Recently cleansed cells were adjusted to 1×10^6 cells/ml in F5P medium by mixing equal volumes of conditioned F10P medium with F0P medium. Then 6 ml of cells were mixed with a volume of F0P and test article, with or without S-9. The cells were exposed to the mutagen for 4 hours in 5% CO_2-air atmosphere at 37°C on a rollerdrum.

Expression

After a 4-hour exposure, the cells were washed with 2 volumes of F0P medium and then resuspended in 20 ml of F10P medium, gassed with 5% CO_2-air atmosphere and then incubated overnight at 37°C. For the following 3 days, the mutated cells were given the opportunity to express the mutation by being passed daily at 3×10^5 cells/ml. On the third day, the cells were counted and cloned in soft-agar medium.

Cloning

The cells were cloned in two types of soft-agar medium, with TFT for detection of TK-/- mutants and without TFT for evaluation of cloning efficiency. The cloning media for detection of the TK-/- mutants consisted of 20% v/v horse serum, 69.5% v/v Fischer's medium, 0.5% v/v (0.2 mg/ml) TFT solution, and 10% v/v (4.0% w/v agar in distilled water) agar solution. Four replicates were made for each dose. The cells for each dose were dispensed at 1×10^6 in the complete TFT medium and dispensed in a 100 x 15 mm petri dish. The petri dishes were then exposed to 4°C for 10 minutes after which they were placed in an incubator at 37°C in 5% CO_2-air atmosphere for 11 days. All procedures were conducted in a room lit with non-UV yellow light. For testing the cloning efficiency of the cells, similar cloning media and procedures were used, omitting only the TFT. The number of cells plated was 300 cells/petri dish.

Counting Colonies and Data Analyses

The cell colonies were counted manually with the aid of a Fisher Acculite Colony Counter and/or Zoom stereomicroscope. Colony numbers among the four replicates within each dose-test article group were examined for possible outliers using the non-parametric test for outliers (CRC Handbook, 1968). The level of significance for acceptance or rejection of an outlier was $p < 0.05$. Several outliers were detected and are identified in the relevant data tables. These outliers were not used further. In no instance was there more than one outlier per group, and the number of such outliers was relatively small. After elimination of the outliers the remaining colony numbers were used to compute means and standard deviations for each dose-test article group. Data analyses were conducted using the MINITAB Data Analyses Software (State College, PA) on our in-house Data General MV8000 computer.

It was necessary to test whether the test articles were cytotoxic, as evidenced by a reduction in numbers of colonies, and further, to establish the dose-cytotoxic response relationship. To this end, two parallel studies were conducted simultaneously for each test article. The first was a "viability" test (without TFT) in which numbers of colonies were counted, while the second was a "mutagenicity" test (with TFT) in which the numbers of observed mutated clones were scored. There were thus two separate studies: a dose-cloning study (viability) and a dose-mutation study (mutagenicity).

In the mouse lymphoma assay when TFT is added to the medium, only mutant colonies can grow. If a test article inhibited or interfered with colony formation, i.e., had a detrimental effect on cloning efficiency, it would be

expected that fewer colonies, and hence, fewer mutant colonies would be seen. Since a range of dose-levels of the test articles were used the cytotoxic effect would vary from level to level. It is necessary to calculate the number of colonies that would have been present if there were no toxicity. The assumption here is that cloning efficiency is independent of the addition of TFT. Thus the data from the viability test are used to normalize the numbers of colonies in the mutagenicity test to constant numbers of colonies at all dose levels.

The ease with which such normalization may be effected depends upon the dose-cytotoxicity relationship. In the simplest case the relationship is linear, and the normalization of the mutant numbers may be readily performed by using the ratios of each dose-cytotoxicity estimated value to its estimated control value. Thus a dose that reduced viable colonies to 50% of the negative controls would require that the number of mutants in the corresponding dose-mutagenicity study be doubled.

In experiments IIA (Table 2) and IIB (Table 3) the concordance between viability and mutation was poor at higher dose levels. It was not possible to utilize regression analyses to normalize the numbers of mutations. Normalization in these instances was effected by multiplying the numbers of mutations at each dose level by the ratio: viability of the negative controls divided by the viability at that dose level. In these analyses, the basis for viability is the mean of the four replicates of the negative controls. In experiment III (Table 6) it was possible to utilize all the data in a regression analysis to estimate the negative control viability.

The results of the dose-cytotoxicity studies, in terms of the numbers of viable colonies on each replicate plate, were utilized in a simple, linear regression analysis. The significance of the regression coefficient was tested using the Analysis of Variance (ANOVA) resulting from the regression analysis. The F statistic (mean square regression/mean square error) had 1 and n-2 degrees of freedom, where n=total number of replicate plates. The F statistic tested the null hypothesis that b, the regression coefficient, was equal to zero. Equivalently, the t-test where $t=b/S_b$ would also test whether $b=0$. Here S_b is the standard deviation of b.

In a dose-cytotoxicity or dose-mutagenicity study with 5 dose levels, for example, there would be 20 replicate plates (5x4). There would, then, be 18 degrees of freedom with which to test that $b=0$. Should $b\neq 0$ and should the square of the correlation coefficient, r, be large, the larger part of the variation among colony numbers is accounted for by the relationship with dose, and normalizations to account for cell losses due to toxicity can be made. Additionally, a large r^2 means that there is little evidence against non-linearity between dose and cytotoxicity or dose and mutagenicity.

Table 4 contains data to illustrate the methods used. For DEGDN (top of table) the regression analysis produced the equation:

$$Y = 206 - 814X$$

where the Y are the numbers of colonies observed in replicate plates treated with dose X of DEGDN. The regression coefficient, b, = -814, indicated that cell numbers declined with increasing dose of DEGDN, and was highly significantly different from zero ($P < 0.005$). The correlation coefficient $r = 0.77$ was also significant. Its square, $r^2 = 0.59$, indicates that the association between cloning efficiency and dose accounted for 59% of the variation in colony numbers. For DIGL-RP and the JA-2 (Table 4), the r (and r^2) were much higher, due to less variation among replicate plates.

The DEGDN regression equation for cloning efficiency was $\hat{Y} = 206 - 814X$. We calculate the estimated colony numbers ($\hat{Y}$) using this equation:

X (dose)%	Y (observed)	$\hat{Y}$ (estimated)	$\hat{Y}$ dose/$\hat{Y}$ control
0.0	210	206	1.00
0.021	195	189	0.917
0.042	156	172	0.835
0.085	145	137	0.665

The observed mean numbers of mutants were normalized by dividing them by the ratios in the last column above, for comparable doses. For DEGDN, in this study, the mean mutant numbers were (see Table 6):

Dose, %	Observed Mean Mutants	Normalizing Factor	Normalized Mutants
0.0	18.3	1.00	18.3
0.021	19.5	0.917	21.3
0.042	21.3	0.835	25.5
0.085	21.0	0.665	31.6

The means of the normalized mutants were regressed against dose producing:

$$Y = 18.3 + 159X$$

with $r = 0.998$, $p < 0.001$.

The criteria for a valid test include the following conditions: 1) adequate negative solvent controls, 2) adequate positive controls, and 3) a mimimum of three test doses plus 0 dose. In a positive result, the test article induces mutation frequency with a positive dose response that is statistically significant at $p \leq 0.01$ and/or when the test article induces a two-fold or more increase in mutation frequency.

RAUSCHER LEUKEMIA VIRUS-INFECTED RAT EMBRYO CELL (RLV-RE)

The Fischer F344 rat embryo culture chronically infected with Rauscher leukemia virus ($2FR_450$) was developed by Traul et al.[14] and kindly supplied by J. Rhim (NCI) at passage 9. These cells were expanded to passage

15 and frozen in multiple vials at 4 x 10^6 cells/ml. As recommended, the cells were removed from the flask surface using saline-trypsin-versene solution (STV) and were passaged at 2.6 x 10^4 cells/cm^2 in Eagle's media supplemented with 10% v/v heat inactivated fetal bovine serum, 0.29 mg/ml L-glutamine, 0.1 mg/ml gentamicin, 50 units/ml penicillin and 50 µg/ml streptomycin.

RLV-RE CELL TRANSFORMATION ASSAY

Cytotoxicity Test

RLV-RE cells were seeded into 24-well plates at 2.6 x 10^4 cells/cm^2. The plates were incubated for 48 hours at 37°C in 5% CO_2-air atmosphere. After the cells were in active growth phase, the cells were exposed to the test agents and permitted to incubate for 7 days at 37°C in 5% CO_2-air atmosphere. After 7 days the cells were washed, fixed with methanol and stained with Giemsa stain. The cytotoxic effect of the test articles based on the number of adherent cells per given area, was determined by microscopic examination and compared to the control to establish the LD_{50} for each test agent. The final assay included one concentration at LD_{50} and one at marginal toxic concentration where the number of adherent cells were estimated to be 90-95% of the control.

Test Exposure

RLV-RE cells at passage 15 were seeded into 25 cm^2 tissue culture flasks at 2.6 x 10^4 cells/cm^2 and permitted to proliferate for 48 hours at 37°C in 5% CO_2-air atmosphere. After 48 hours the cells were exposed to the test articles in duplicate and with positive (3-MCA) and negative (solvent) controls. The exposed RLV-RE cells were permitted to incubate for 7 days at 37°C in 5% CO_2-air atmosphere. The cells were washed free of the test article with serum free medium and then fresh maintenance medium was added to each flask.

Expression

The expression period allows the genotoxic damage to the cells to become phenotypically evident as cell transformation. Freeman et al. (1973) found that subdivision or passaging of the cell culture was necessary for the manifestation of cell transformation. During the expression period, half of the cell culture from each group was exposed to the tumor promoter, TPA. TPA was added to enable the detection of a weak carcinogen or of initiators which are dependent on initiator-promoter interaction for the expression of cell transformation.[11]

During the expression period, the cell cultures were fed twice a week with fresh maintenance medium. Two weeks after the exposure period, the cells from each 25 cm^2 flask were passed to 75 cm^2 and designated as P-1. Two weeks later P-1 cells were subdivided 1:3 and designated P-2 cells. One of the 3 flasks became the "holding" flask to be stained for detection of morphological alteration after 3 weeks. The other 2 flasks were used for passaging. The

cells in 1 of 2 flasks was exposed to 0.25 µgm/ml TPA. In subsequent passages the cells that were with and without TPA were subdivided 1:3 and designated as P-3 holding, passage and reserve: the holding flasks were examined for morphologic alteration after staining; the passage flasks were passed 1:3 after 2 weeks; and reserve flasks were used if one of the other flask was lost by contamination. Cell cultures at passage 4 through 6 were treated identically as P-3 except the cells in the reserve flasks were used for cloning in semi-solid agar media for detection of transformed cells with anchorage-independent growth.

Cloning in Semi-Solid Agar Media

In order to detect transformed cells with anchorage-independent growth, cells from P-4 and P-6 were cloned in semi-solid agar media. Four replicate flasks for each exposure were prepared by seeding 1 x 10^6 cells/75 cm^2 flask in Eagle's Minimum Essential media supplemented with 20% v/v fetal bovine serum, 0.36% agar, 0.1 mg/ml gentamicin, 50 units/ml penicillin and 50 µg/ml streptomycin.

Counting Colonies and Data Analysis

After examining the stained "holding" flasks, the decision was made not to rely on morphological transformation as an endpoint since the determination and number of transformed foci appeared to be very subjective. Therefore, the cell transformation data were based on colony counts of cells capable of continual proliferation in semi-solid agar media. The colonies were counted with aid of a stereo dissecting microscope. Since limited growth was observed in negative controls in negative controls, ranges of 0-23 with 2% DMSO and 4-40 with 2% DMSO + acetone, the flasks were examined for clonal growth at 3, 4 and 5 weeks to confirm that the transformed cells showed continual growth.

One-way analysis of variance (ANOVA) was used to determine whether the number of colonies produced by the test samples were significantly different from that of the negative control. A valid test must have included a negative solvent control, a positive control and three doses including 0 dose. RLV-RE cells are known for low spontaneous transformation. Ideally, the concentration of the positive control should induce a 10-fold increased transformation above background. The test article was considered to have produced a positive result if one or both of the following criteria was met:

(1) A statistically significant ($P \leq 0.01$) increase in transformation frequency, or

(2) A 2-fold or greater increase above control in the numbers of colonies per plate.

RESULTS

MUTAGENICITY

The data from two dose-range experiments for the mutagenicity of the three test articles, DEGDN, DIGL-RP and JA-2, with and without rat liver S-9 activation are summarized in Tables 2 and 3. The number of viable colonies (VC), which represented the survivors of the effects of the test articles, are shown in column % VC, the number of counted mutants (CM)/10^6 cells are in column CM and the normalized mutants (NM)/10^6 cells are in column NM. The normalized number of mutants were calculated by the following formula: NM = (control VC/dose VC) X CM (see Methods).

The first two experiments IA and IIA (Table 2) were conducted to determine the cytotoxic and mutagenic activities of the test articles, respectively without S-9 activation. DEGDN was tested at 4 concentrations between 0.011 and 0.085% v/v while DIGL-RP was tested at 7 concentrations between 6.25 and 200 µg/ml and JA-2 was tested at 6 concentrations between 6.25 and 200 µg/ml. Negative controls for the solvents were included as was a single positive control (EMS) as a direct-acting mutagen to demonstrate that the assay was functional. The viability results of Experiment IA (Table 2) indicated that DEGDN did not affect viability adversely, but that DIGL-RP, at doses of 25 µg/ml or higher, and JA-2 at doses of 25 µg or higher, severely reduced viability. Thus in the design of Experiment IIA the highest doses used for DIGL-RP and JA-2 were 50 µg/ml.

Because of highly toxic effects of DIGL-RP and JA-2 on MLC at the concentrations tested in experiment IA, the experiment was repeated but at lower concentrations for DIGL-RP and JA-2. In experiment IIA, the normalized mutant values for the three test articles suggested a positive weak dose-mutation response for DEGDN and stronger dose-mutation response for DIGL-RP and JA-2. The regression analyses for the three test articles indicated significant positive dose-mutation correlations for DEGDN, DIGL-RP and JA-2 ($r=0.80$ for DEGDN, $r=0.99$ for DIGL-RP, and $r=0.96$ for JA-2). When the toxicity of DIGL-RP and JA-2 reduced VC counts below 50% of controls, it was observed that the number of microcolonies, i.e., colonies at the lower limits of scoring (and enumeration) as a colony, increased sharply. With increasing numbers of microcolonies the enumeration of those cell clusters as to whether they were colonies or non-colonies became more subjective and the actual colony counts became more variable. While Experiment IIA showed a positive dose-mutation response it was considered worthwhile to perform another experiment to evaluate the test articles at less toxic concentrations.

In assessing the three test articles for the presence of a promutagen through the use of rat liver S-9 activation, we evaluated the test articles at the same concentrations as the previous experiments (IA and IIA). The data for two experiments are summarized on Table 3. As in the previous experiments without activation, we included solvent controls and single positive promutagen 2-AAF which required rat liver S-9 activation for mutagenic activity to confirm that the cells were responsive. In dose-range experiment IB with activation, DEGDN was again tested at 4 concentrations between 0.011 and 0.085% v/v while DIGL-RP and JA-2 were tested at 5 and 4 concentrations between 25 and 200 µg/ml, respectively. At concentrations greater than 50 µg/ml, a precipitate was observed after DIGL-RP and JA-2 were added to the reaction mixture. Because of precipitation and poor cell growth, the cells were plated only for mutants.

TABLE 2. MOUSE LYMPHOMA CELL FORWARD MUTATION ASSAY DOSE-RANGE EXPERIMENTS WITHOUT S-9 ACTIVATION.

Compound	Dose	Experiment IA Mutants±SD[a] CM	Experiment IIA % VC[b]	Mutants±SD[a] CM	NM[a]
Control	0[c]	10.8± 2.2	100	18.5± 3.9	18.5± 3.9
DEGDN	0.011% v/v	12.3± 5.7	100	16.8± 4.4	16.8± 4.4
DEGDN	0.021% v/v	22.2± 5.7	68	16.3± 4.3	23.8± 6.3
DEGDN	0.042% v/v	19.5± 2.1	93	20.3± 6.6	21.8± 7.1
DEGDN	0.085% v/v	15.0± 8.3	32	14.5± 3.1	45.0± 9.6
				Y = 15.00 + 318.90X	
				r = 0.80, p < 0.001	
Control	0[d]	6.0± 1.2	100	21.3±1.0	21.3± 1.0
DIGL-RP	6.25 µg/ml	N.D.	100	53.8±14.2	53.8± 14.1
DIGL-RP	12.50 µg/ml	N.D.	50	37.7±10.1	69.8± 18.7
DIGL-RP	25.00 µg/ml	44.0±11.0	35	66.3±24.0	189.4± 68.6
DIGL-RP	50.00 µg/ml	41.8±10.7	7	30.8±11.9	440.0±170.0
DIGL-RP	100.00 µg/ml	10.0± 4.7			
DIGL-RP	150.00 µg/ml	1.5± 1.0		Y = -6.2 + 8.59X	
DIGL-RP	200.00 µg/ml	0.5± 0.6		r = 0.99, P < 0.001	
Control	0[d]	6.0± 1.2	100	21.3± 1.0	21.3± 1.0
JA-2	6.25 µg/ml	N.D.	100	26.5± 8.3	26.5± 9.6
JA-2	12.50 µg/ml	N.D.	45	48.8±17.9	137.1± 49.7
JA-2	25.00 µg/ml	33.0± 4.5	23	42.8±12.5	244.5± 71.4
JA-2	50.00 µg/ml	13.5±13.5	0	26.5± 9.0	
JA-2	100.00 µg/ml	2.5± 3.0		Y = 1.9 + 9.64X	
JA-2	200.00 µg/ml	0		r = 0.96, P < 0.01	
EMS[e]	2.5 mM	235.3±39.2		295.8±30.5	

a Mutants ± SD = mutants/10^6 cells ± standard deviation.
CM = Counted Mutants, NM = Normalized Mutants.
NM calculated as follows: NM = (Control VC/dose VC) X CM.

b % VC = % viable colonies.

c Control = 4.0% v/v DMSO/0.015% acetone.

d Control = 4.0% v/v DMSO.

e The direct-acting mutagen EMS served as the positive control for each experiment to demonstrate that the cells were responsive.

TABLE 3. MOUSE LYMPHOMA CELL FORWARD MUTATION ASSAY DOSE-RANGE EXPERIMENTS WITH S-9 ACTIVATION.

		Experiment IB	Experiment IIB		
Compound	Dose	Mutants±SD[a] CM	% VC[b]	Mutants±SD[a] CM	NM[a]
Control	0[c]	12.8± 2.6	100	25.5±3.7	25.5± 3.7
DEGDN	0.011% v/v	12.3± 4.6	100	47.8±4.0	47.8± 4.0
DEGDN	0.021% v/v	16.8± 8.4	100	35.2±1.7	35.2± 1.7
DEGDN	0.042% v/v	16.3± 3.0	92	31.0±4.3	33.7± 4.3
DEGDN	0.085% v/v	9.8± 3.1	80	29.8±6.1	37.2± 6.1
				Y = 34.90 + 31.40X	
				r = 0.00, N.S.[g]	
Control	0[d]	10.5± 1.7	100	27.8±3.2	27.8± 3.2
DIGL-RP	6.25 µg/ml	N.D.	100	37.0±5.2	37.0± 5.2
DIGL-RP	12.50 µg/ml	N.D.	97	33.0±4.2	34.0± 4.3
DIGL-RP	25.00 µg/ml	21.3±12.9	97	29.0±6.3	29.9± 6.5
DIGL-RP	50.00 µg/ml	14.5± 3.7[e]	93	18.3±3.3	19.7± 3.5
DIGL-RP	100.00 µg/ml	26.8±10.3			
DIGL-RP	150.00 µg/ml	16.6± 1.3		Y = 34.52 - 0.26X	
DIGL-RP	200.00 µg/ml	21.8± 8.3		r = -0.58, p < 0.01	
Control	0[d]	10.5± 1.7	100	27.8±3.2	27.8± 3.2
JA-2	6.25 µg/ml	N.D.	69	22.5±5.2	32.6± 7.5
JA-2	12.50 µg/ml	N.D.	79	24.8±8.6	31.4±10.9
JA-2	25.00 µg/ml	25.3± 1.3	83	21.8±7.5	26.3± 9.0
JA-2	50.00 µg/ml	23.5± 8.4[e]	65	19.3±7.5	29.7±11.5
JA-2	100.00 µg/ml	29.0± 2.2		Y = 29.88 - 0.023X	
JA-2	200.00 µg/ml	20.0± 6.8		r = 0.00, N.S.[g]	
2-AAF[f]	50.00 µg/ml	295.8±30.5		211.8±18.6	

a Mutants±SD = mutants/10^6 cells ± standard deviation.
CM = Counted Mutants, NM = Normalized Mutants.
NM calculated as follows: NM = (Control VC/dose VC) X CM.

b % VC = % viable colonies.

c Control = 4.0% v/v DMSO/0.015% v/v acetone.

d Control = 4.0% v/v DMSO.

e Reaction mixtures greater than 50 µg/ml formed precipitation that affected cell growth and count.

f The promutagen 2-AAF served as the positive control for each experiment to indicate that the cells were responsive.

g Not significant.

In the presence of rat liver S-9 the three test articles produced on altered response from that seen when no S-9 was added (compare Tables 3 and 2). As noted earlier a precipitate had formed when S-9 was added to plates containing 50 µg/ml or more of DIGL-RP or JA-2. Since the precipitation interfered with the first experiment, the experiment (IIB) was repeated at lower concentrations to determine whether the test articles which had mutagenic activity without rat liver S-9 induced enhanced mutagenic activity in the presence of rat liver S-9 as an indication for the possible presence of a promutagen. DEGDN was tested at the same concentrations between 0.011 and 0.085% v/v while DIGL-RP and JA-2 were tested at 4 concentrations between 6.25 and 25 µg/ml. The results indicated that the addition of rat liver S-9 reduced or neutralized the toxic and mutagenic effects of the test articles at concentrations found to contain direct-acting mutagen(s). The normalized mutagenic activity of the three test articles, DEGDN, DIGL-RP and JA-2, with and without activation are plotted as histograms on Figures 1, 2, and 3, respectively. The comparative mutagenicity of the three test articles with and without activation indicated that the mutagenic activity was lowered with the addition of the activator. However, mutant numbers were not correlated with increased dose. Regression analyses and analyses of variance of the data for the rat liver S-9 activated dose-mutation experiments on DEGDN and JA-2 were found not to be statistically significant. DIGL-RP had a negative dose-mutation correlation ($r=-0.58$) which was statistically significant ($p < 0.01$) but not biologically significant as a promutagen. This suggested that the three test articles contained a direct-acting mutagen and contained no promutagen detectable by this test system.

To confirm that the three test articles contained a direct-acting mutagen(s), we repeated the experiment without rat liver S-9 activation (experiment III). In previous experiments where the test articles were highly toxic to the MLC, it was observed that there were increased numbers of microcolonies, which were more difficult to count. Thus, experiment III was conducted at lower concentrations for DIGL-RP and JA-2, for DIGL-RP and JA-2, while three levels of DEGDN were used (0.021, 0.042, and 0.085%). The data for experiment III are in Tables 4, 5 and 6 which include dose-viability data, dose-mutation data and the viable counts and normalized mutants, respectively. Statistical analysis of the plating efficiency showed that an increasing concentration of the test articles had an inverse effect on the plating efficiency for each test article (upper regression lines Figures 4-6). The toxic effects of DEGDN, DIGL-RP and JA-2 on MLC produced negative linear correlations between dose and cell viability ($r=-0.77$ for DEGDN, -0.90 for DIGL-RP, and -0.95 for JA-2) and these values were statistically significant ($p < 0.005$ for DEGDN, $p < 0.001$ for DIGL-RP and JA-2). This suggested that cells exposed to the test articles were irreparably damaged and failed to grow as efficiently in semi-solid agar medium as the control cells. To compensate for the dose-effect damage on clonal growth, we normalized the mutant values. The normalized mutant data (Table 6) clearly indicated linear dose-mutation correlations for DEGDN, DIGL-RP, and JA-2 with positive coefficients of correlation ($r=0.998$ for DEGDN, 0.994 for DIGL-RP and 0.995 for JA-2). These correlations are statistically significant ($p < 0.001$). The regression lines for the test articles are plotted in Figures 4, 5, and 6 (lower regression lines).

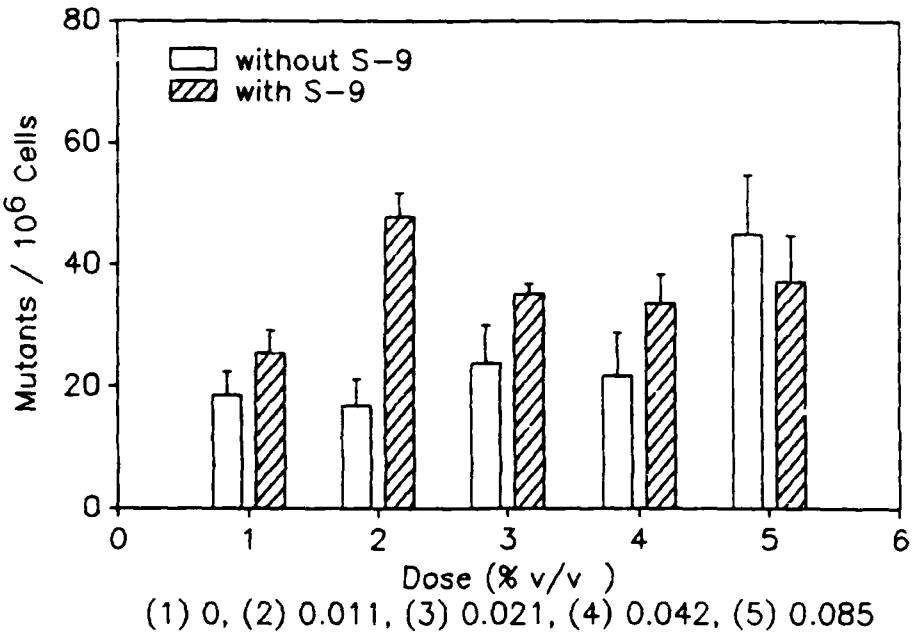


Figure 1. Histogram comparing normalized mutation induced by DEGDN with and without rat liver S-9 activation.

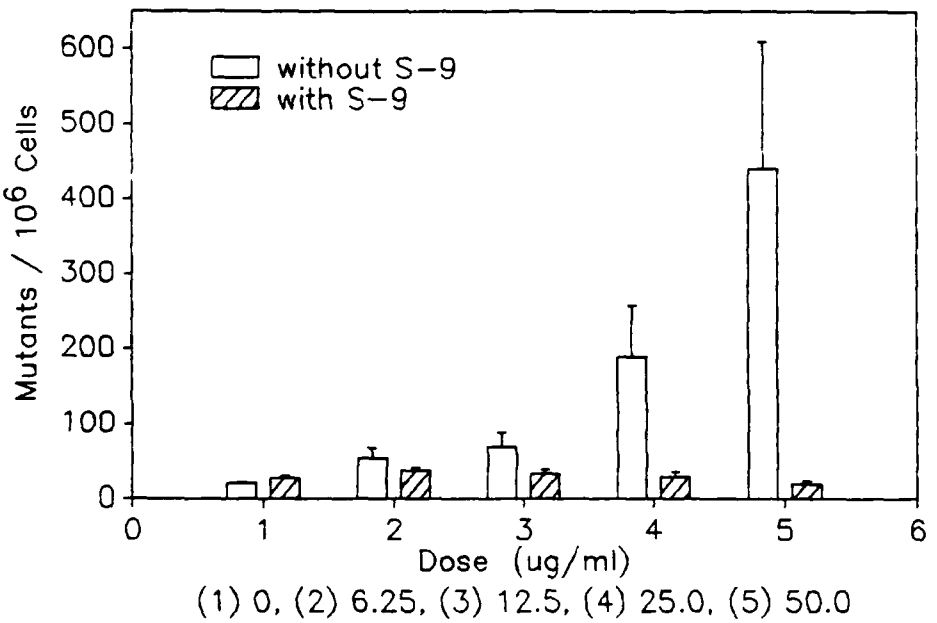


Figure 2. Histogram comparing normalized mutation values induced by DIGL-RP with and without rat liver S-9 activation.

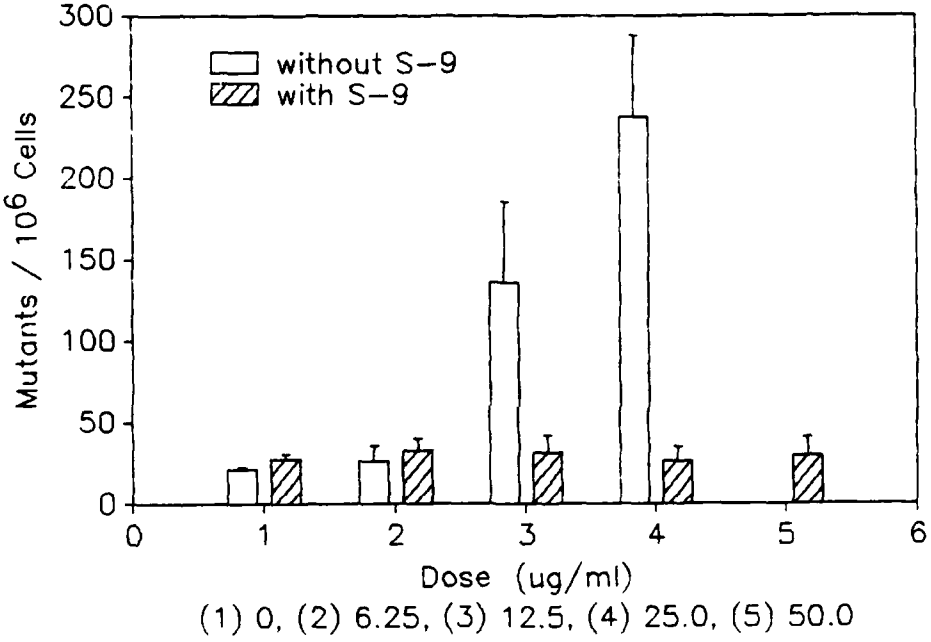


Figure 3. Histogram comparing normalized mutation values induced by JA-2 with and without rat liver S-9 activation.

TABLE 4. MOUSE LYMPHOMA CELL FORWARD MUTATION ASSAY VIABLE COLONY DATA FOR EXPERIMENT III WITHOUT S-9 ACTIVATION.

Compound	Dose	Replicate No. of Colonies				Mean	SD	% Survivors
Control	4.0% v/v DMSO/ 0.015% v/v Acetone	227	215	188	210	210	16.3	100
DEGDN	0.021%	209	160	215	122*	195	30.2	93
DEGDN	0.042%	164	186	149	124	156	26.1	74
DEGDN	0.085%	146	147	143	115*	145	2.1	69
Regression equation Y = 206 - 814X		Coefficient of correlation r = -0.77					Significance p < 0.005	
Control	4.0% v/v DMSO	213	206	198	195	203	8.1	100
DIGL-RP	1.00 µg/ml	169	155	140	162	157	12.4	77
DIGL-RP	3.12 µg/ml	140	186	168	125	155	27.4	76
DIGL-RP	6.25 µg/ml	135	139	133	98*	136	3.1	67
DIGL-RP	12.50 µg/ml	110	133	87	62*	110	23.0	54
DIGL-RP	25.00 µg/ml	69	56	77	60	66	9.4	33
Regression equation Y = 175 - 4.64X		Coefficient of correlation r = -0.90					Significance p < 0.001	
Control	4.0% v/v DMSO	213	206	198	195	203	8.1	100
JA-2	1.00 µg/ml	187	185	196	156*	189	5.9	93
JA-2	5.00 µg/ml	156	165	155	157	158	4.6	78
JA-2	10.00 µg/ml	123	111	115	92	110	13.2	54
JA-2	20.00 µg/ml	88	79	89	88	86	4.7	42
Regression equation Y = 192 - 5.88X		Coefficient of correlation r = -0.95					Significance p < 0.001	
EMS	2.5 mM	136	148	146	170	150	14.3	

* Outlier not used in calculation.

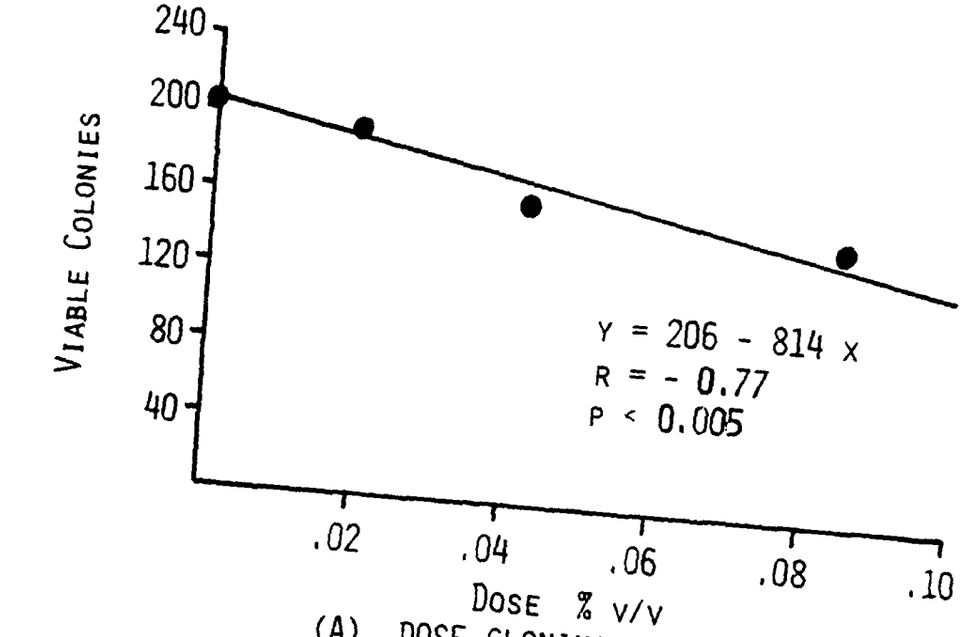
TABLE 5. MOUSE LYMPHOMA CELL FORWARD MUTATION ASSAY MUTAGENICITY DATA FOR EXPERIMENT III WITHOUT S-9 ACTIVATION.

Compound	Dose	Replicate No. of Colonies				Mean	SD
Control	4.0% v/v DMSO/ 0.015% v/v Acetone	17	18	21	17	18.3	1.9
DEGDN	0.021%	12	22	18	26	19.5	6.0
DEGDN	0.042%	25	14	25	21	21.3	5.2
DEGDN	0.085%	20	27	20	17	21.0	4.2
Control	4.0% v/v DMSO	14	20	13	22	17.3	4.4
DIGL-RP	1.00 µg/ml	11	25	18	20	18.5	5.8
DIGL-RP	3.12 µg/ml	45	50	38	39	43.0	5.6
DIGL-RP	6.25 µg/ml	43	48	40	45	44.0	3.4
DIGL-RP	12.50 µg/ml	71	55	61	70	64.3	7.6
DIGL-RP	25.00 µg/ml	50	62	47	49	52.0	6.8
Control	4.0% v/v DMSO	14	20	13	22	17.3	4.4
JA-2	1.00 µg/ml	12	12	17	20	15.3	4.0
JA-2	5.00 µg/ml	33	30	32	30	31.3	1.5
JA-2	10.00 µg/ml	46	44	40	40	42.5	3.0
JA-2	20.00 µg/ml	38	38	35	38	37.3	1.5
EMS	2.5 mM	179	150	135	152	154	18.3

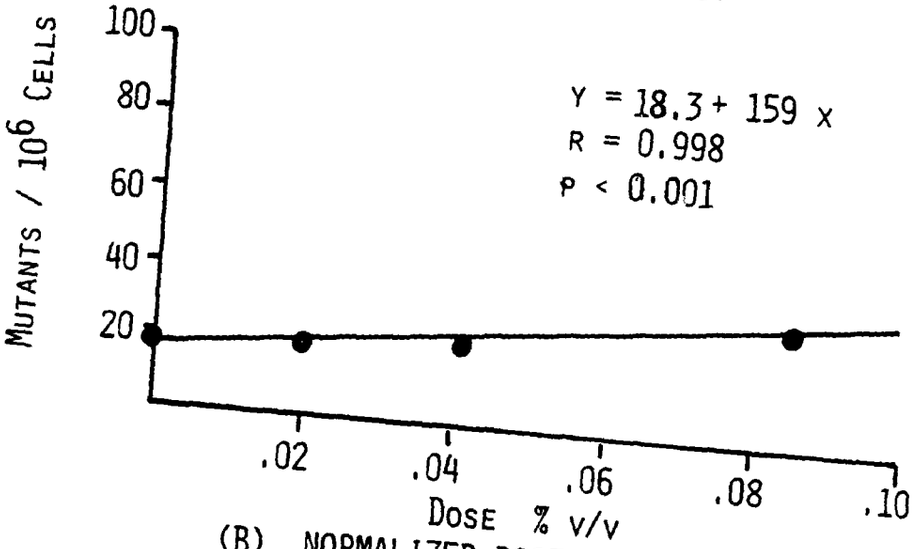
TABLE 6. MOUSE LYMPHOMA CELL FORWARD MUTATION ASSAY SUMMARY OF VIABILITY AND MUTAGENICITY DATA FROM DOSE-MUTATION EXPERIMENT III OF DEGDN, DIGL-RP AND JA-2 WITHOUT S-9 ACTIVATION.

Compound	Dose	Viable Colonies Mean±SD	% Survivors	Mutants/10^6 Cells Mean±SD	Normalized*
Control	4.0% v/v DMSO/0.015% v/v Acetone	210±16.3	100	18.3±1.9	18.3
DEGDN	0.021%	195±30.2	93	19.5±6.0	21.3
DEGDN	0.042%	156±26.1	74	21.3±5.2	25.5
DEGDN	0.085%	145± 2.1	69	21.0±4.2	31.6
Regression equation Y = 18.3 + 159X		Coefficient of correlation r = 0.998			Significance p < 0.001
Control	4.0% v/v DMSO	203± 8.1	100	17.3±4.4	17.3
DIGL-RP	1.00 µg/ml	157±12.4	77	18.5±5.8	19.0
DIGL-RP	3.12 µg/ml	155±27.4	76	43.0±5.6	46.9
DIGL-RP	6.25 µg/ml	136± 3.1	67	44.0±3.4	52.7
DIGL-RP	12.50 µg/ml	110±23.0	54	64.3±7.6	96.0
DIGL-RP	25.00 µg/ml	66± 9.4	33	52.0±6.8	154.2
Regression equation Y = 20.2 + 5.47X		Coefficient of correlation r = 0.994			Significance p < 0.001
Control	4.0% v/v DMSO	203± 8.1	100	17.3±4.4	17.3
JA-2	1.00 µg/ml	189± 5.9	93	15.3±4.0	15.7
JA-2	5.00 µg/ml	158± 4.6	78	31.3±1.5	36.9
JA-2	10.00 µg/ml	110±13.2	54	42.5±3.0	61.3
JA-2	20.00 µg/ml	86± 4.7	42	37.3±1.5	96.1
Regression equation Y = 15.8 + 4.12X		Coefficient of correlation r = 0.995			Significance p < 0.001
Positive Control EMS	2.5 mM	150±14.3	74	154±18.3	208.1

* See methods section for calculation.



(A) DOSE-CLONING EFFECT



(B) NORMALIZED DOSE-MUTATION EFFECT

FIGURE 4. REGRESSION LINES FOR DEGDN: (A) DOSE-CLONING EFFECT AND (B) NORMALIZED DOSE-MUTATION EFFECT. THE LINES WERE DETERMINED FROM THE DOSE-MUTATION EXPERIMENT III, TABLES 4 AND 6.

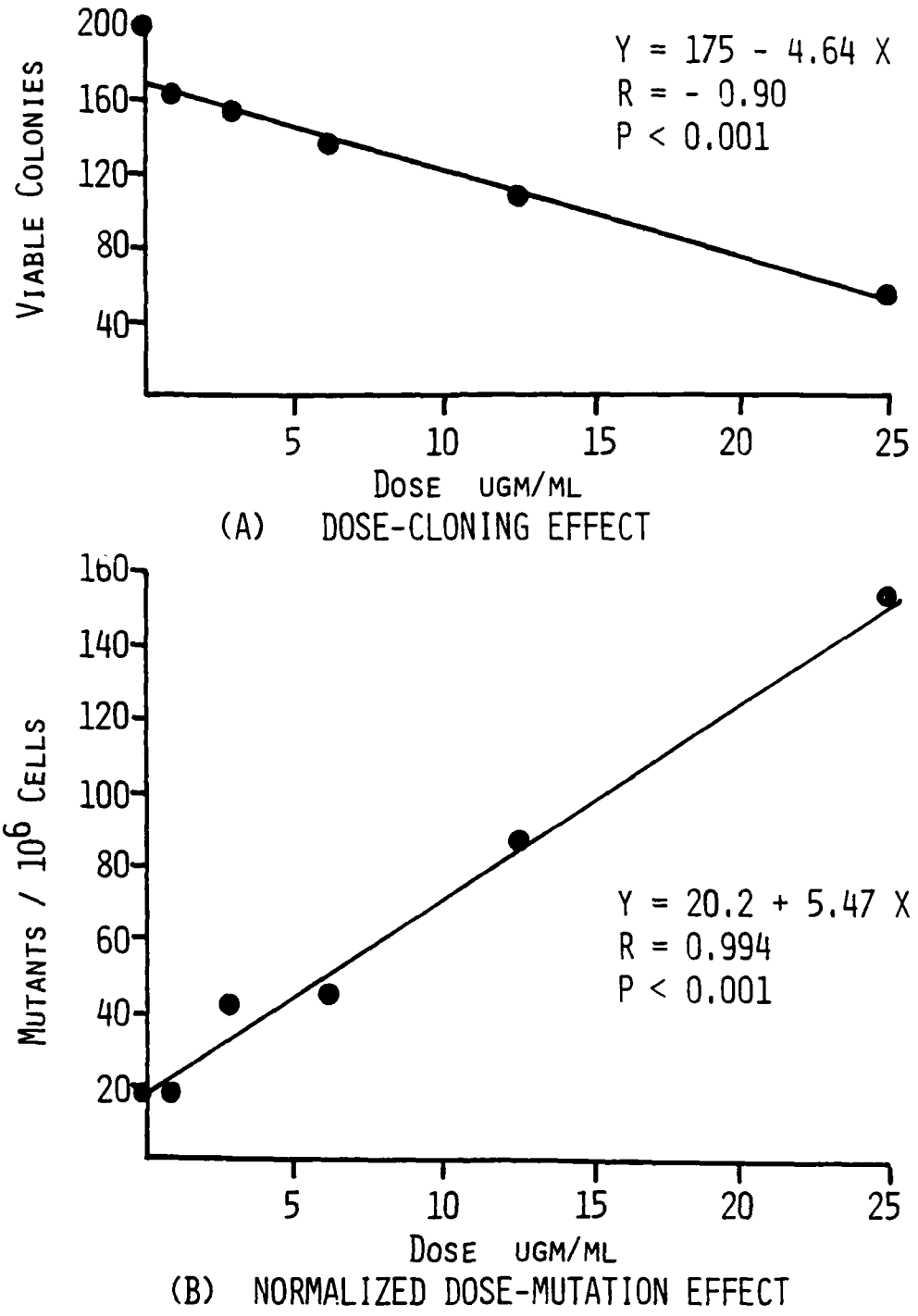
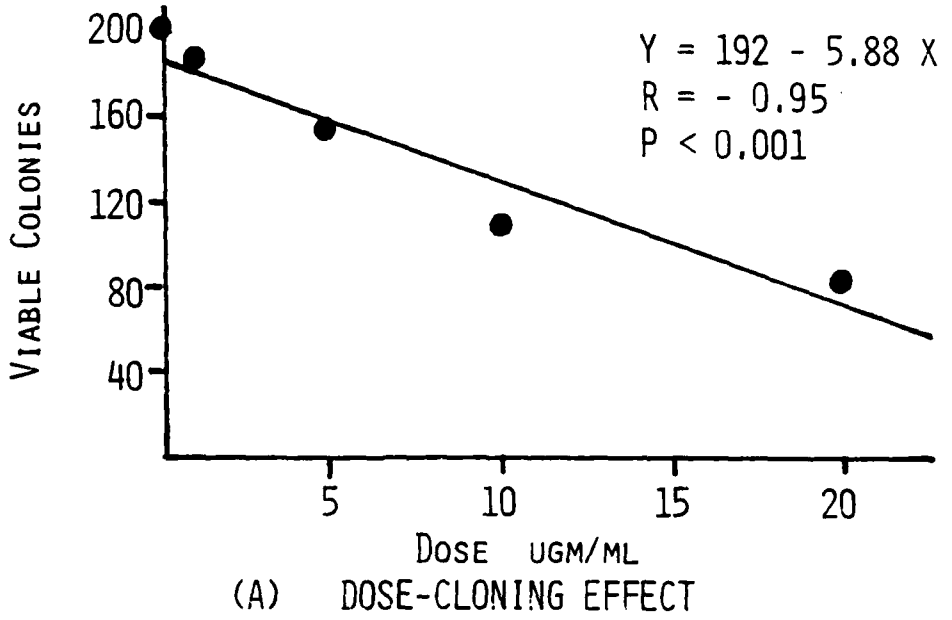
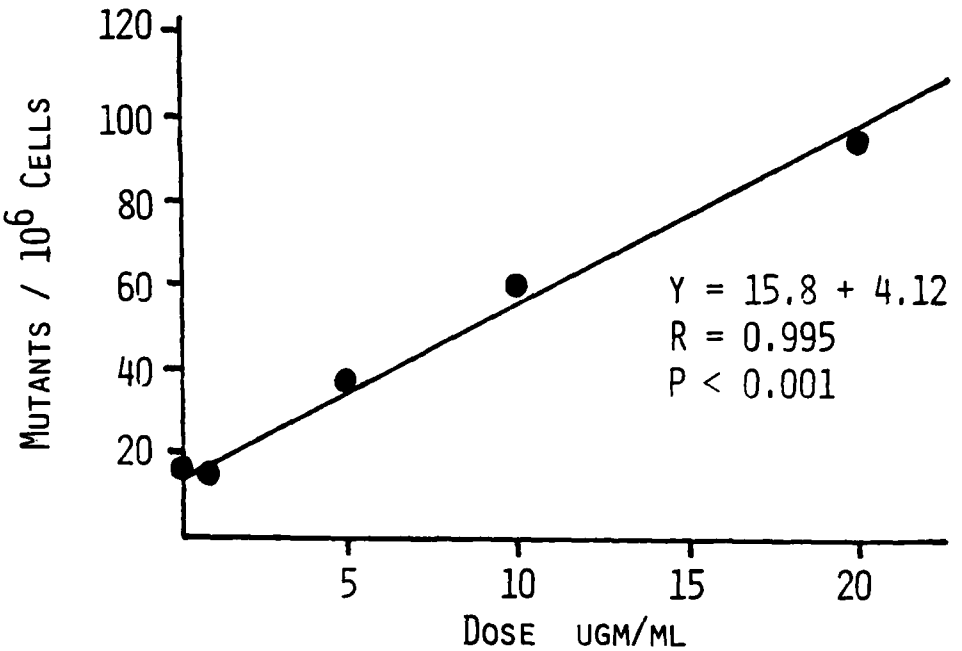


FIGURE 5. REGRESSION LINES FOR DIGL-RP: (A) DOSE-CLONING EFFECT AND (B) NORMALIZED DOSE-MUTATION EFFECT. THE LINES WERE DETERMINED FROM THE DOSE-MUTATION EXPERIMENT III, TABLES 4 AND 6.



(A) DOSE-CLONING EFFECT



(B) NORMALIZED DOSE-MUTATION EFFECT

FIGURE 6. REGRESSION LINES FOR JA-2: (A) DOSE-CLONING EFFECT AND (B) NORMALIZED DOSE-MUTATION EFFECT. THE LINES WERE DETERMINED FROM THE DOSE-MUTATION EXPERIMENT III, TABLES 4 AND 6.

These experiments indicated that the three test articles contained a direct-acting mutagen(s) and the mutagenic activity was not enhanced by activation. Based on the mutagenic activity detected by the mouse lymphoma cell mutagenicity assay, DEGDN appeared to be a weak mutagen because it demonstrated a statistically significant linear dose-mutation response and only approached or barely exceeded a 2-fold increase in numbers of mutants above background. On the other hand, both DIGL-RP and JA-2 were strongly mutagenic based on the two criteria for a positive mutagen.

In order to confirm that the cells that grew in TFT agar medium were true mutants, we removed numerous cell clones from the TFT medium of the DIGL-RP and JA-2 dose-mutation experiments and permitted the TFT-resistant mutants to proliferate for over 20 generations in TFT-free medium. Since the experiment was to assess whether the cells capable of proliferating in soft-agar plates with TFT were true mutants, mutant colonies were taken at random from JA-2 and DIGL-RP exposed plates. The source of the cells was not recorded. After the TFT-resistant mutants were passaged in TFT-free medium for several weeks, the cells (8×10^4 cells/ml) in complete medium that was supplemented with 200 µgm/ml methotrexate or 100 µgm/ml TFT were evaluated for growth after 4 days. Table 7 summarizes the data this test. The data indicate that TFT-resistant mutants continued to be resistant to TFT but sensitive to methotrexate, confirming that the cells that grew in TFT medium were true mutants.

TABLE 7. QUALITY CONTROL TEST FOR MUTANTS.

Clone	Cell Growth in Methotrexate Media x 10^6 Cells/ml	Cell Growth in Trifluorothymidine Media x 10^6 Cells/ml
1	0.18	2.20
2	0.11	1.50
3	0.05	0.93
4	0.14	1.80
5	0.15	1.90
6	0.16	2.40
7	0.15	1.80
8	0.17	1.50
9	0.12	2.30

Each tube seeded with 8×10^4 cells/ml and counted in a cell counter (Coulter, model ZB-1) after 4 days. The clones were isolated from TFT medium of the DIGL-RP and JA-2 dose-mutation experiment.

IN VITRO CARCINOGENICITY

After the mutagenicity tests clearly indicated that the 3 test articles had mutagenic activity in mammalian cells, we initiated the RLV-RE cell transformation assay to establish whether the three test articles were also potential carcinogens. In the RLV-RE transformation assay, where the transformed cells have anchorage-independent growth characteristics, the transformed cells have an increased tendency to become readily detached from the plastic flask. In this assay, where the cells were washed and fed twice weekly with fresh medium, and passed at biweekly intervals, there was an increased tendency to lose the transformed cells during these manipulations. The number of transformed cells that may be lost during any of these manipulation can vary and affect the final transformed cell counts. While the counted colonies represented transformed cells, the final judgment as to relative potency of the carcinogen, based on the number of colonies, is at best an estimated value.

The test articles were each tested at three doses, at three dose levels; DEGDN at 0, 0.022, and 0.045%, and DIGL-RP and JA-2, each at 0, 50 and 100 µg/ml. The results of RLV-RE cell transformation with and without a promoter are summarized in Table 8. Of the RLV-RE cells exposed to the three test articles without TPA, cells exposed to DIGL-RP at 50 and 100 µg/ml consistently had increased numbers of transformed colonies at both 4th and 6th cell-passage level. One group, DIGL-RP at 50 µg/ml had an unusually high number of colonies (3863 and 6900) while the other two plates in this group had smeared colonies which could not be counted. The reason for the high count is not known, although it is possible that the cells were in the process of acquiring anchorage-independent growth characteristics, thus had not become readily detached and lost during the twice weekly manipulation. The other test articles failed to show a positive response by the 6th passage. Statistical analysis (one way analysis of variance) of the data for DIGL-RP indicated that transformation were statistically significant ($p < 0.01$). Although the positive control had lower transformed colonies than expected, there was a positive response above background at the 6th cell passage. Since cells exposed to carcinogens require several passages before expressing transformation characteristics, it is possible that further passages could have increased the number of transformed colonies.

While the RLV-RE cell transformation assay is usually performed without the addition of a promoter, we included TPA for the possible detection of an initiator or weak carcinogen. The test articles were tested at the same concentrations as the previous experiment without TPA. The results are also summarized on Table 8. With the addition of TPA, we observed that DEGDN with or without TPA was not effective in enhancing cell transformation, DIGL-RP with or without TPA was equally effective in inducing cell transformation, while JA-2 with TPA was effective in inducing cell transformation but not in the absence of TPA. However, a problem was encountered with the positive control where we observed early anchorage-independent transformation, before the fourth passage, which caused the transformned cells to become detached , the non-transformed cells remain attached to the flask, and lost during cell feeding. This tended to favor the non-transformed cells to remain in the culture. The early expression of anchorage-independent transformation and

TABLE 8. CARCINOGENICITY OF DEGDN AND TWO DEGDN-CONTAINING COMPOUNDS EVALUATED BY RLV-RE CELL TRANSFORMATION ASSAY.

Compound		Without Promoter 12-O-Tetradecanoyl phorbol 13-acetate (TPA) Anchorage-Independent Growth			
		Passage 4		Passage 6	
		Mean* Colonies/ 10^6 cells	SD	Mean* Colonies/ 10^6 cells	SD
Control	DMSO/Acetone	10.3	2.9	13.5	9.3
DEGDN	0.022% v/v	5.0	2.8	16.8	3.2
DEGDN	0.045% v/v	1.8	2.1	4.0	2.8
Control	DMSO 2% v/v	14.3	3.6	14.3	3.6
DIGL-RP	50 µg/ml	5382**	--	281.8	73.4
DIGL-RP	100 µg/ml	289	107	216.8	49.3
JA-2	50 µg/ml	2.8	1.0	19.3	4.5
JA-2	100 µg/ml	2.3	3.2	10.8	5.1
3-MCA	0.5 mM	17.0	3.5	66.0	21.6

Compound		With Promoter (TPA) Anchorage-Independent Growth			
		Passage 4		Passage 6	
		Mean* Colonies/ 10^6 cells	SD	Mean* Colonies/ 10^6 cells	SD
Control	DMSO/Acetone	1.0**	**	27.5	11.3
DEGDN	0.022% v/v	1.5	0.6	6.5	2.7
DEGDN	0.045% v/v	0.7	0.6	15.8	4.2
Control	DMSO 2% v/v	0.0	0.0	17.5	6.1
DIGL-RP	50 µg/ml	572.5	92.5	486.5	270.6
DIGL-RP	100 µg/ml	***	***	0.0	0.0
JA-2	50 µg/ml	2.0	2.7	12.0	3.6
JA-2	100 µg/ml	42.0	55.2	567.0	31.1
3-MCA	0.5 mM	1.0	0.6	1.3	1.5

* Mean of 4 plates, except as noted.
** Mean of 2 plates.
*** Colonies were smeared and could not be counted.

loss of the transformed cells during feeding of the cells may partially explain the low number of transformed cells in the positive control. In the absence of a positive response with the positive control, the results obtained with DIGL-RP and JA-2 were unequivocal. However, RLV-RE cells are known for their low spontaneous transformation. Since the negative controls failed to have high transformation cell counts, the results obtained with DIGL-RP and JA-2 suggested that these two test articles were very likely responsible for the results. One way analysis of variance of the data for DIGL-RP and JA-2 indicated that the results are statistically significant ($p < 0.01$). The present results are in agreement with the mutagenicity study, where both DIGL-RP and JA-2 appear to be potential mutagen-carcinogens. DEGDN is probably not a carcinogen, based on the results in Table 8.

DISCUSSION

The present study indicates that the three test articles, DEGDN, DIGL-RP, and JA-2 have mutagenic activity in mammalian cells in vitro. The results indicate that DEGDN is a weak mutagen while DIGL-RP and JA-2 are strong mutagens. Under identical experimental conditins DEGDN, with and without the promotor TPA failed to transform RLV-RE cells, while DIGL-RP with and without TPA was capable of transforming RLV-RE cells. JA-2 with TPA, but not without TPA, was able to transform RLV-RE cells. Although DEGDN is a common component of both DIGL-RP and JA-2, the mutagenic activity that was observed with these test articles appears not to be from DEGDN. Since the test articles DIGL-RP and JA-2 are complex mixtures, the specific mutagenic activity was not determined. However, if one compares the relative mutagenic activity of the three nitrate ester-containing samples based on the total weight of the three test articles, DIGL-RP and JA-2 are 355-and 269-fold, respectively, more active than DEGDN (Table 9). Although this study appears to be the first to report mutagenic-carcinogenic activity of a nitrate ester-containing compound in mammalian cells in vitro, MacPhee et al.[15] have reported nitroglycerin to be mutagenic in bacterial cells. Whether these nitrate ester-containing test articles are mutagenic in bacterial cells is not known.

TABLE 9. SUMMARY OF RELATIVE MUTAGENIC/CARCINOGENIC ACTIVITY OF DEGDN AND TWO DEGDN CONTAINING SOLID PROPELLANTS.

Compound	Mutagenicity	Carcinogenicity (Cell Transformation)
DEGDN	Weak direct-acting mutagen *R.M.A. = 1 mutant/65 µg/ml = 1 mutant/117 µM	Negative with or without TPA
DIGL-RP	Strong direct-acting mutagen R.M.A. = 1 mutant/0.183 µg/ml = 1 mutant/0.51 µM	Positive with or without TPA
JA-2	Strong direct-acting mutagen R.M.A. = 1 mutant/0.242 µg/ml = 1 mutant/0.64 µM	Positive with TPA Negative without TPA

* R.M.A. = relative mutagenic activity of a test agent, calculated from the experimental data, is the quantity of test agent in µg/ml or µM, necessary to induce one mutant.

While there is no report that ethyl centralite (N N'-diethyl-N N' diphenylurea) or N-methyl-N' N' diphenylurea are mutagens, a number of reports indicate that several urea compounds are mutagens. Thust, et al.[16], reported that 1,3-dimethyl-3-phenyl-1-nitrosourea was found to induce mutation in bacterial and mammalian cells. Lee, et al.[17] tested 22 nitrosourea compounds with the Ames assay and reported that several of the compounds were mutagens. If the urea compound is in fact the mutagen, it constitutes a very small percentage of the total weight of DIGL-RP and JA-2, suggesting that the neat compound is a very strong mutagen, or possibly, there is a strong synergistic effect of several weak mutagens in the complex mixture, accounting for the strong mutagenic activity associated with DIGL-RP and JA-2. The capacity of DIGL-RP and JA-2 to induce cell transformation of RLV-RE cells strongly supports the results of the mutagenicity assay. The capacity of DIGL-RP to induce cell transformation by the fourth passage without TPA also agrees with the mutagenicity assay, and suggests that DIGL-RP is a potent mutagen/carcinogen. The detection of putative carcinogenic activity of JA-2 in the presence of TPA may be similar to reported transformations in RLV-RE cells at subeffective doses of chemical carcinogen in the presence of a tumor promoter.[11]

In conclusion, these studies indicate that DEGDN is a weak mutagen and that DIGL-RP and JA-2 are strong mutagens, and that DIGL-RP and JA-2 are also potential carcinogens. Since DIGL-RP and JA-2 are complex mixtures, further studies are needed to identify the specific active mutagen/carcinogen(s).

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DATA COLLECTION AND STORAGE

Raw data which were manually entered into the hardbound laboratory book and the final report are stored in Room 431, Bldg. H-215, at L.E.H.R., University of California, Davis, California 95616. The storage area is under the control of the Quality Assurance Officer.

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DAVIS, CALIFORNIA 95616

October 31, 1988

THE FOLLOWING STUDY: Evaluation of Diethyleneglycoldinitrate (DEGDN) and Two DEGDN-Containing Compounds was inspected by the LEHR Quality Assurance Unit and reported to management on the following dates:

INSPECTION DATES	REPORT TO MANAGEMENT
7/31/85	8/30/85
8/13/85	8/30/85
8/29/85	8/30/85
12/19/85	1/14/86
1/9/86	1/14/86
2/26/86	3/18/86
3/19/86	5/8/86
12/23/86	12/23/86
8/15/87	8/15/87
10/30/88	10/31/88

Jennifer J. Short Date 10/31/88

Jennifer J. Short
CPRC Quality Assurance

PERSONNEL

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T. Kawakami, Ph.D.

Co-Investigators: A. Aotaki-Keen, B.S.
L. S. Rosenblatt, Ph.D.
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Veterinary Services: Gary Cain, DVM, Ph.D.

Statistician: Leon S. Rosenblatt, Ph.D.

Quality Assurance Unit: Jenny Short

SIGNATURE PAGE

Final Report for Study Number 4856, APO No. 84PP4856, July 1987

Evaluation of Diethyleneglycoldnitrate (DEGDN)
and Two DEGDN Containing Compounds

Prepared by _____ 7/9/88
M. Goldman, Ph.D., Study Director — Date

and _____ 7/9/88
T. G. Kawakami, Ph.D., Principal Investigator — Date

Reviewed by _____ July 10, 1988
L. S. Rosenblatt, Ph.D., Biostatistician — Date

Reviewed by _____ 7/10/88
Gary Cain, DVM, Ph.D., Veterinarian — Date

Reviewed by _____ 10/31/88
Jenny Short, Manager, Quality Assurance Unit — Date

Reviewed by _____ 11/2/88
Otto G. Raabe, Acting Director, LEHR — Date

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PROTOCOL #1

Determination of Cell Mutagenicity and Transformation in Mammalian Cells exposed to Certain Nitrate Ester Materials

Project Order #84PP4856

Principal Investigator:

Dr. Thomas G. Kawakami

Laboratory for Energy-Related Health Research
University of California
Davis, California

October 10, 1984

Prepared for the

US Army Medical Bioengineering
Research and Development Laboratory
Fort Detrick, Maryland

The current proposal is the last task in Phase I. Three nitrate ester materials will be tested in the project. One compound is Diethyleneglycoldinitrate (DEGDN) as manufactured to meet MIL-SPEC-DOD-D-64015, 14 June 1983. The other two compounds are propellant mixes utilizing DEGDN: JA2 (MIL-SPEC-DOD-P-63493, 14 June 1983) and DIGL-RP (MIL-SPEC-DOD-P-63492, 30 June 1983). These three compounds will be evaluated by the Mouse Lymphoma Mutation Assay and the Cell Transformation Assay. The specific locus muational assay system for mouse lymphoma cells (L5178Y) is an assay to evaluate mutagens in mammalian somatic cells. Chemical transformation of retrovirus-infected rat embryo cells from several rat strains infected with non-transforming rat retro-virus and in which chemical carcinogens enhance cell transformation of these cells. The data from these studies indicate a good correlation between the ability of chemicals to transform cells in vitro and their known carcinogenic potential in animals.

Task 1: Mouse Lymphoma (L5178Y/$TK^{+/-}$) Forward Mutation Assay

The specific locus mutational assay system for mouse lymphoma cells (L5178Y) is an assay to evaluate mutagens in mammalian somatic cells. By selecting a gene locus that can be readily detected, we can quantitate the effect of mutagens on a specific gene locus. Thymidine kinase (TK) is an enzyme that is encoded by a specific gene locus which is involved in synthesis of thymidine monophosphate (TMP). TMP occupies a unique position in DNA synthesis because it does not undergo significant conversion to other nucleotides. The cellular TMP pool is small and depletion of the pool can arrest DNA synthesis. Replacement of TMP in the DNA with a lethal TMP analogue can result in the death of the cell. 5-Bromodeoxyuridine (BUdR) monophosphate and trifluorothymidine (TFT) monophosphate are lethal analogues of TMP which result from phosphorylation of BUdR or TFT by the "salvage" enzyme TK. Mutant cells which are deficient in TK activity ($TK^{-/-}$) lack the enzyme and thus do not suffer any ill effect when they are exposed to BUdR or TFT. By selecting mutant cells that are heterozygous for the thymidine kinase gene locus ($TK^{+/-}$) which is sensitive to BUdR or TFT, one can measure the forward mutation at that gene locus, e.g. $TK^{+/-}$ -- $TK^{-/-}$ by counting the number of cloned colonies that form in medium containing BUdR or TFT following exposure to mutagens. Following a standardized procedure with adequate controls, one can quantitate the mutagenic potential of various compounds. Three months are estimated to perform this research for each "unknown."

Mouse Lymphoma Mutation Study

Personnel		Salary and Benefits
Oncologist		$ 3,540
Research Technician		10,300
	Total	13,840
Supplies and Expenses		
Tissue culture, glassware, etc.		2,400
Media		960
Chemicals		960
	Total	4,260
Total Direct Costs		18,100
Laboratory Services @35%		6,335
Total Laboratory Costs		24,435
UC Overhead @18.5%		4,520
TOTAL TASK COST		$27,955

Task 2: Cell Transformation Assay for Chemical Carcinogens with Retrovirus-infected Rat Embryo Cells

Chemical transformation of retrovirus-infected rat embryo cells is a technique that uses rat embryo cells from several rat strains infected with non-transforming rat retrovirus and in which chemical carcinogens enhance cell transformation of these cells. These cells transplanted in syngeneic hosts or nude mice were found tumorigenic while control cells failed to induce tumors. The data from these studies indicate a good correlation between the ability of chemicals to transform cells in vitro and their known carcinogenic potential in animals. This technique will be adapted to evaluate the transforming potential of the DMSO "fractions" or unknowns.

Rat embryo cell cultures are plated out (15 ml of 10ES cells) in 75 cm^2 T flasks. The culure is chronically infected with non-transforming rat leukemia virus (RLV). On day 7, the cultures are subdivided 1:2. On day 8, the cultures are treated with the candidate chemical, the concentration is predetermined by a cytotoxicity assay (see below). The chemical transformation potential is determined at 5- and 10-fold concentrations above and below the TD_{50} cytotoxicity level. Four replicate cultures are treated at each concentration. On day 11, each of the 4 cultures is subdivided 1:2 in the presence of chemical, which is then removed on day 15. After the chemical is removed, one of the two daughter cultures is subdivided every second week (vertical series), while the other is held for 60 days. The cell cultures from the vertical series are passed up to 6 times. All the cultures are fed 3 times per week with fresh medium. Prior to each passage, the cultures are examined for foci of cell transformation, and on passage the cells are cloned in soft agar medium to evaluate for attachment-free growth. Chemically exposed cells that show foci of transformed cells or have colony growth in soft agar will be considered positive chemical carcinogen. All cultures that are positive for cell transformation will be evaluated for tumorigenicity in syngeneic host or nude mice to confirm that the chemical is a carcinogen (see below).

A cytotoxicity assay is initially conducted for each chemical by adding graded concentrations to 1000 embryo cells that had been plated out 24 hours earlier. After a 7-day exposure to the chemical, including a medium refeed on day 3, the cells are stained with crystal violet. Cell colonies with 8 or more cells are counted and the median toxic dose (TD50) is calculated based on the cloning efficiency of the control culture.

Malignant transformation is confirmed by transplanting 10E6 cells subcutaneously into newborn isogeneic rats or nude mice. The animals are maintained for a 6-month period to assure the tumorigenicity of the transformed cells. Thus this evaluation bridges the short term cell mutagenesis determination and the long term whole animal carcinogenesis measurement.

The advantages of the retrovirus infected rat embryo transformation assay are that a large number of compounds have been evaluated and the published data indicate a good correlation between the ability of chemicals to transform cells in vitro and their known carcinogenic potential in animals The procedure is more labor and space intensive and requires long periods of time for examination than simple mutagenesis assays. The transformation potential of a chemical carcinogen can not be quantitated per cell in this assay but it does represent a major step prior to determination of dose-effect relations.

The initial set-up time will vary because a pool of frozen, low passage rat embryo cell cultures must be initially established. After the cells are infected with RLV, the virus-infected embryo cells must be standardized and characterized for spontaneous transformation and for effectiveness of known chemical to induce cell transformation. The estimated time line for the first compound is 12 months and thereafter, the time line for additional compounds will be 6 months. Since several compounds can be staggered during the 6-month period, the time needed will depend on whether single or multiple compounds are to be evaluated.

The cost for the first compound will be higher because of the time, material, and labor required for standardization and characterization of the cell transformation assay. However, after the assay is on-line the cost will be about 1/3 for each additional compound relative to the first unknown.

Mammalian Cell Transformation Study

Personnel		Salary and Benefits
Oncologist		$ 5,900
Research Technician		20,600
	Total	26,500
Supplies and Expenses		
Tissue culture, glassware, etc.		3,600
Media		1,000
Chemicals		950
	Total	5,550
Total Direct Costs		32,050
Laboratory Services @35%		11,218
Total Laboratory Costs		43,268
UC Overhead @18.5%		8,005
Total task cost		$51,273
Total Cost for both tasks		$80,128

LEHR will follow GLP procedures. As a part of research on chemical agent material for this Laboratory, LEHR is scheduled to be inspected for GLP compliance by the Food and Drug Administration in the near future.

Reporting requirements will adhere to USAMBRDL "Contractor Reporting Requirements" for the Health Effects Research Division (March 1984). Bimonthly progress reports are required.

PROTOCOL #2

PROTOCOL FOR TESTING MUNITION COMPOUNDS DEGDN, JA-2, DIGL

DEGDN, diethylene glycol dinitrate, yellowish viscous liquid, 15% acetone.

JA-2 fine powder pulverized at Letterman, contains (% by weight) 59.5% nitrocellulose, 24.8% DEGDN, 14.9% nitroglycerin, 0.7% N-methyl-N'-N' Diphenylurea, 0.04% graphite, 0.04% magnesium oxide and maximum of 0.2% graphite glaze.

DIGL fine powder pulverized at Letterman, contains (% by weight) 62.5% nitrocellulose, 36.7% DEGDN, 0.25% ethyl centralite, 0.45% methyl diphenylurea, 0.04% graphite, 0.04% magnesium oxide and maximum of 0.2% graphite glaze.

A. Concentrations to test the above compounds:

1. DEGDN. Since this compound contains 15% acetone and is very toxic to cells, this dictated the top concentration. Dr. Kawakami recommended testing 0.015% acetone which has shown some toxicity. Thus, the highest concentration to test will be at 0.085% DEGDN.

2. JA-2 and DIGL. These two compounds are currently being tested at Letterman where we had the compounds pulverized. Their highest stock solution is 200 mg/ml and therefore we followed their recommendation.

B. Initial toxicity test shows:

1. JA-2 is more soluble than DIGL.

2. 0.2 mg/ml of both JA-2 and DIGL is barely soluble even if the DMSO concentration is at 4%.

3. 0.015% acetone is still somewhat toxic and should be used as the highest concentration when considering the testing range of DEGDN.

4. Maximum concentration (final concentration) to be tested are 0.2 mg/ml of both JA-2 and DIGL and 0.085% DEGDN.

5. Minimum concentration to be tested are 0.025 mg/ml of both JA-2 and DIGL and 0.011% DEGDN.

6. All the DMSO in this test will be kept constant at 4% final concentration.

We plan to start testing these compounds July 28, 1985. The first test will be of these compounds without enzyme activation (without S-9). The results of this first test is then due in 2 weeks. The second test will involve testing these compounds at the same concentrations with enzyme activation. This will be done in early August.

Further tests will be planned after studying the results of these tests.

Testing Munition Compounds without Liver Enzyme

Cells are adjusted to 1 x 10^6 cells/ml in 5% serum Fisher's media.

JA-2 and DIGL are dissolved and diluted in DMSO.

DEGDN is dissolved in 40% DMSO solution.

100 µl of DEGDN (which is 85% DEGDN, 15% Acetone) with 4 ml of DMSO.

Then add 5.9 ml FOP.

This is the highest concentration working solution.

This is further diluted using a solution that is 40% DMSO, 60% FOP.

Solvent Control for DEGDN is made with 40% DMSO, 0.15% Acetone, 59.85% FOP.

Tube No.	Description	Amount Compound and Concentration	Amt FOP	Amount Cells
0	No Add	0	4 ml	6 ml
100	4% DMSO	0.4 ml DMSO	3.6 ml	6 ml
101	JA-2 200 µg/ml	0.4 ml (5 mg/ml) JA-2	3.6 ml	6 ml
102	100 µg/ml	0.4 ml (2.5 mg/ml) JA-2	3.6 ml	6 ml
103	50 µg/ml	0.4 ml (1.25 mg/ml) JA-2	3.6 ml	6 ml
104	25 µg/ml	0.4 ml (0.625 mg/ml) JA-2	3.6 ml	6 ml
105	DIGL 200 µg/ml	0.4 ml (5 mg/ml) DIGL	3.6 ml	6 ml
106	150 µg/ml	0.4 ml (3.75 mg/ml) DIGL	3.6 ml	6 ml
107	100 µg/ml	0.4 ml (2.5 mg/ml) DIGL	3.6 ml	6 ml
108	50 µg/ml	0.4 ml (1.25 mg/ml) DIGL	3.6 ml	6 ml
109	25 µg/ml	0.4 ml (0.625 mg/ml) DIGL	3.6 ml	6 ml
110	4% DMSO, 0.15% Acetone	1 ml (40% DMSO, 0.15% Acetone)	3 ml	6 ml
112	DEGDN 0.085%	1 ml (0.85%) DEGDN	3 ml	6 ml
113	0.0425%	1 ml (0.425%) DEGDN	3 ml	6 ml
114	0.022%	1 ml (0.22%) DEGDN	3 ml	6 ml
115	0.011%	1 ml (0.11%) DEGDN	3 ml	6 ml
150	EMS 2.5 nM	100 µl (250 mM) EMS	4 ml	6 ml

Testing Munition Compound With Liver Enzyme

To make the cofactor mix, weigh:

0.960 gm isocitric acid
0.512 gm NADP

Dissolve in 42.7 ml FOP and neutralize with 1N NaOH. Filter through 0.22 µm millex type filter. The above weight of cofactors (isocitric and NADP) would usually be dissolved in 64 ml of FOP. In this experiment, however, the compound concentration from the usual 1% to 10% and 4%. Thus the FOP volume is reduced and adjusted.

To make the S-9 mix, add:

20 ml of liver S-9
40 ml of cofactor mix
Keep on ice until ready to use.

Cells are adjusted to 1 x 10^6 cells/ml in 5% serum Fisher's media.

Tube No.	Description	Amount Compound and Concentration	Amt S-9 Mix	Amt FOP	Amt Cells
0	S-9 No-Add	0	3 ml	1 ml	6 ml
200	4% DMSO	0.4 ml DMSO	3 ml	0.6 ml	6 ml
201	JA-2 200 µg/ml	0.4 ml (5 mg/ml) JA-2	3 ml	0.6 ml	6 ml
202	100 µg/ml	0.4 ml (2.5 mg/ml) JA-2	3 ml	0.6 ml	6 ml
203	50 µg/ml	0.4 ml (1.25 mg/ml) JA-2	3 ml	0.6 ml	6 ml
204	25 µg/ml	0.4 ml (0.625 mg/ml) JA-2	3 ml	0.6 ml	6 ml
205	DIGL 200 µg/ml	0.4 ml (5 mg/ml) DIGL	3 ml	0.6 ml	6 ml
206	150 µg/ml	0.4 ml (3.75 mg/ml) DIGL	3 ml	0.6 ml	6 ml
207	100 µg/ml	0.4 ml (2.5 mg/ml) DIGL	3 ml	0.6 ml	6 ml
208	50 µg/ml	0.4 ml (1.25 mg/ml) DIGL	3 ml	0.6 ml	6 ml
209	25 µg/ml	0.4 ml (0.625 mg/ml) DIGL	3 ml	0.6 ml	6 ml
210	4% DMSO, 0.15% Acetone	1 ml (40% DMSO, 0.15% Acetone)	3 ml	0 ml	6 ml
212	DEGDN 0.085%	1 ml (0.85%) DEGDN	3 ml	0 ml	6 ml
213	0.0425%	1 ml (0.425%) DEGDN	3 ml	0 ml	6 ml
214	0.022%	1 ml (0.22%) DEGDN	3 ml	0 ml	6 ml
215	0.011%	1 ml (0.11%) DEGDN	3 ml	0 ml	6 ml
250	2AAF 50 µg/ml	100 µl (5,000 µg/ml) 2AAF	3 ml	1 ml	6 ml
251	100 µg/ml	100 µl (10,000 µg/ml) 2AAF	3 ml	1 ml	6 ml

Issue date 9/3/85

LABORATORY FOR ENERGY-RELATED HEALTH RESEARCH

UNIVERSITY OF CALIFORNIA

DAVIS, CALIFORNIA

*** PROTOCOL ADDENDUM ***

STUDY NUMBER APOH4856. PROTOCOL NUMBER 072885

STUDY TITLE Mouse lymphoma Assay on Certain Nitrate Ester Mater

ADDENDUM NUMBER 1

REASON(S) FOR CHANGE Some of the doses previously tested proved toxic. Lowered the dose concentrations.

STUDY DIRECTOR APPROVAL [signature] DATE 10-5-85

EFFECTIVE DATE OF CHANGE 9-20-85

CIRCULATION LIST:

Addendum to Protocol of Munition Testing by Mouse Lymphoma Cells

Results of tne initial testing showed that munition compounds have mutagenic activity. However, the viability of cloning or the cloning efficiency was very poos in spite of seemingly good growth in the liquid culture. To rectify this difference and to verify the mutagenicity, repeat the test with the following changes:

1) When making dilutions for the viability cloning, do not treat the cells with trypsin (to dissociate the cells) as the cells to be counted are treated by trypsin. Possibility that some of the groups have weakend membrane or damaged structure and cannot withstand treatment with trypsin.

2) Even though the viability was poor at the higher concentrations of JA-2 and DIGL and it may be due to trypsin and not the chemicals themselves, there was no mutation since the TFT agar growth was also very poor. These cells were not treated by trypsin, thus start as the highest dose to test JA-2 and DIGL as 50 µg/ml.

3) Repeat the DEGDN at the same doses as before.

4) When repeating the S-9 portion, it may be necessary to mix the S-9 mix with the compound and centrifuge to get rid of the precipitate before exposing the S-9/compound mixture to the cells.

5) Doses to be repeated for the S-9 portion will be decided after seeing the test results from the initial test.

Tube No.	Description	Amount Compound and Concentration	Amt FOP	Amount Cells
0	No Add	0	4 ml	6 ml
100	4% DMSO	0.4 ml DMSO	3.6 ml	6 ml
101	JA-2 50 µg/ml	0.4 ml (1.25 mg/ml) JA-2	3.6 ml	6 ml
102	25 µg/ml	0.4 ml (625 µg/ml) JA-2	3.6 ml	6 ml
103	12.5 µg/ml	0.4 ml (312.5 µg/ml) JA-2	3.6 ml	6 ml
104	6.25 µg/ml	0.4 ml (156 µg/ml) JA-2	3.6 ml	6 ml
105	DIGL 50 µg/ml	0.4 ml (1.25 µg/ml) DIGL	3.6 ml	6 ml
106	25 µg/ml	0.4 ml (625 µg/ml) DIGL	3.6 ml	6 ml
107	12.5 µg/ml	0.4 ml (312.5 µg/ml) DIGL	3.6 ml	6 ml
108	6.25 µg/ml	0.4 ml (156 µg/ml) DIGL	3.6 ml	6 ml
109	4% DMSO, 0.15% Acetone	1 ml (40% DMSO, 0.15% Acetone)	3 ml	6 ml
110	DEGDN 0.085%	1 ml (0.85% DEGDN)	3 ml	6 ml
111	0.0425%	1 ml (0.425%) DEGDN	3 ml	
112	0.0425%	1 ml (0.22%) DEGDN	3 ml	6 ml
113	0.022%	1 ml (0.11%) DEGDN	3 ml	6 ml
150	EMS 2.5 nM	100 µl	4 ml	6 ml

LABORATORY FOR ENERGY-RELATED HEALTH RESEARCH

University of California
Davis, California

PROTOCOL ADDENDUM

072885

Study number APO#4856 Protocol number ~~9-385~~

Study title Testing Munition Compound with Mouse lymphoma Assay

Addendum number ~~#1~~ #2 SM 9/16/85

Reason(s) for change: Changing the dose to nontoxic dose, dealing with precipitation of compound. Note attached are with & without S-9

Study director approval Marvin Goldman date 9/18/85

Effective date of change: 9-3-85

Circulation list:

OK cover sheet error in addenda

Instructions: Fill out and attach this form to the description of the protocol change or to the re-issued protocol.

8/19/85

LABORATORY FOR ENERGY-RELATED HEALTH RESEARCH

University of California
Davis, California

PROTOCOL ADDENDUM

Study number APO #4856 Protocol number 072885

Study title Testing Munition Compound with Mouse Lymphoma Assay

Addendum number #2

Reason(s) for change: Changing the dose to non toxic dose and removing precipitate from the test (testing with < 9)

Study director approval _____ date _____

Effective date of change: ~~8/85~~ 9/85 sm (9-3-85

Circulation list:

Instructions: Fill out and attach this form to the description of the protocol change or to the re-issued protocol.

8/19/85

Addendum to Protocol of Munition Testing by Mouse Lympnoma Cells.

Munition Compounds tested with Liver Enzyme

Results of the previous experiments on these munition compounds have shown that the higher concentrations of JA-2 and DIGL are still toxic and is thus necessary to test lower concentrations. Therefore we will test the following final concentrations of JA-2 and DIGL.

1) 50 µg/ml
2) 25 µg/ml
3) 12.5 µg/ml
4) 6.25 µg/ml

The previous experiment have also shown that the addition of liver S-9 increases the precipitation of the compound even at 4% DMSO concentration. Therefore the precipitate will be centrifuged out of the mixture before exposing the compound to the cells. This will be done only with the solvent control (4% DMSO) and the JA-2 and DIGL groups. The rest will be done as usual (per SOP). For those, the cell concentration to be added will be changed from 1×10^6 cells/ml to 3×10^6 cells/ml in order to precipitate as much as possible before the addition of more liquid. Mix 4% compound, 30% S-9 mix, 6% FOP, 40% F5P (made by mixing 1 to 1, volume + volume, F1OP and FOP) leaving 20% cell suspension to be added later. Centrifuge at 1600 RPM to remove the precipitate and use only the supernatant to be mixed with the cells.

DEGDN will be tested again at the same.

ADDENDUM #1

Addendum to Protocol of Munition Testing by Mouse Lymphoma Cells

Results of tne initial testing snowed that munition compounds have mutagenic activity. However, the viability of cloning or the cloning efficiency was very poos in spite of seemingly good growth in tne liquid culture. To rectify this difference and to verify the mutagenicity, repeat the test with the following changes:

1) When making dilutions for the viability cloning, do not treat the cells with trypsin (to dissociate the cells) as the cells to be counted are treated by trypsin. Possibility that some of the groups have weakend membrane or damaged structure and cannot withstand treatment with trypsin.

2) Even though the viability was poor at the higher concentrations of JA-2 and DIGL and it may be due to trypsin and not the chemicals themselves, there was no mutation since the TFT agar growth was also very poor. These cells were not treated by trypsin, thus start as the highest dose to test JA-2 and DIGL as 50 µg/ml.

3) Repeat the DEGDN at the same doses as before.

4) When repeating the S-9 portion, it may be necessary to mix the S-9 mix with the compound and centrifuge to get rid of the precipitate before exposing the S-9/compound mixture to the cells.

5) Doses to be repeated for the S-9 portion will be decided after seeing the test results from the initial test.

Tube No.	Description	Amount Compound and Concentration	Amt FOP	Amount Cells
0	No Add	0	4 ml	6 ml
100	4% DMSO	0.4 ml DMSO	3.6 ml	6 ml
101	JA-2 50 µg/ml	0.4 ml (1.25 mg/ml) JA-2	3.6 ml	6 ml
102	25 µg/ml	0.4 ml (625 µg/ml) JA-2	3.6 ml	6 ml
103	12.5 µg/ml	0.4 ml (312.5 µg/ml) JA-2	3.6 ml	6 ml
104	6.25 µg/ml	0.4 ml (156 µg/ml) JA-2	3.6 ml	6 ml
105	DIGL 50 µg/ml	0.4 ml (1.25 µg/ml) DIGL	3.6 ml	6 ml
106	25 µg/ml	0.4 ml (625 µg/ml) DIGL	3.6 ml	6 ml
107	12.5 µg/ml	0.4 ml (312.5 µg/ml) DIGL	3.6 ml	6 ml
108	6.25 µg/ml	0.4 ml (156 µg/ml) DIGL	3.6 ml	6 ml
109	4% DMSO, 0.15% Acetone	1 ml (40% DMSO, 0.15% Acetone)	3 ml	6 ml
110	DEGDN 0.085%	1 ml (0.85% DEGDN)	3 ml	6 ml
111	0.0425%	1 ml (0.425%) DEGDN	3 ml	
112	0.0425%	1 ml (0.22%) DEGDN	3 ml	6 ml
113	0.022%	1 ml (0.11%) DEGDN	3 ml	6 ml
150	EMS 2.5 nM	100 µl	4 ml	6 ml

ADDENDUM #2

Addendum to Protocol of Munition Testing by Mouse Lympnoma Cells.

Munition Compounds tested with Liver Enzyme

Results of the previous experiments on these munition compounds have shown that the higher concentrations of JA-2 and DIGL are still toxic and is thus necessary to test lower concentrations. Therefore we will test the following final concentrations of JA-2 and DIGL.

1) 50 µg/ml
2) 25 µg/ml
3) 12.5 µg/ml
4) 6.25 µg/ml

The previous experiment have also shown that the addition of liver S-9 increases the precipitation of the compound even at 4% DMSO concentration. Therefore the precipitate will be centrifuged out of the mixture before exposing the compound to the cells. This will be done only with the solvent control (4% DMSO) and the JA-2 and DIGL groups. The rest will be done as usual (per SOP). For those, the cell concentration to be added will be changed from 1×10^6 cells/ml to 3×10^6 cells/ml in order to precipitate as much as possible before the addition of more liquid. Mix 4% compound, 30% S-9 mix, 6% F0P, 40% F5P (made by mixing 1 to 1, volume + volume, F1OP and F0P) leaving 20% cell suspension to be added later. Centrifuge at 1600 RPM to remove the precipitate and use only the supernatant to be mixed with the cells.

DEGDN will be tested again at the same.

ADDENDUM #3

Addendum to Protocol for Testing Munition Compounds by Mouse Lymphoma Cells

Repeat mutagenicity test of DIGL, JA-2, and DEGDN using the mouse lymphoma assay without liver enzyme (S-9) activation.

Proposed starting date: May 6, 1986

Proposed ending date: June 1986

Doses to be tested:

DIGL	25 µg/ml, 12.5 µg/ml, 6.25 µg/ml, 3.1 µg/ml, 1 µg/ml
JA-2	20 µg/ml, 10 µg/ml, 5 µg/ml, 1 µg/ml
DEGDN	0.085%, 0.0425%, 0.022%